



IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of:) Examiner: Basi, Nirmal Singh
Avi ASHKENAZI, et al.)
Application Serial No. 09/909,088) Art Unit: 1646
Filed: July 18, 2001) Confirmation No: 1981
For: **SECRETED AND TRANSMEMBRANE**) Attorney's Docket No. GNE-1618 P2C79
POLYPEPTIDES AND NUCLEIC ACIDS)
ENCODING SAME) Customer No. 77845
)

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ON APPEAL TO THE BOARD OF PATENT APPEALS AND INTERFERENCES
APPELLANTS' BRIEF

MAIL STOP APPEAL BRIEF - PATENTS

Commissioner for Patents -
P.O. Box 1450
Alexandria, Virginia 22313-1450

Dear Sir:

On February 1, 2007, the Examiner made a Final rejection to pending Claims 39-47 and 49-52 and 55-58. A response and Notice of Appeal were filed on July 25, 2007. An Appeal Brief was subsequently filed on November 21, 2007.

In an Office Action mailed April 8, 2008, the Examiner withdrew the finality of the previous office action and declared prosecution reopened. As the pending claims had been twice rejected, Applicants filed a further Notice of Appeal on September 8, 2008.

Appellants hereby appeal to the Board of Patent Appeals and Interferences from the last decision of the Examiner. This Brief is timely filed requesting a two-month extension of time with fees.

A supplemental amendment and response to final office action is concurrently filed with the present brief.

The following constitutes Appellants' Brief on Appeal.

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1. REAL PARTY IN INTEREST

The real party in interest is Genentech, Inc., South San Francisco, California, by an assignment of the parent application, U.S. Patent Application Serial No. 09/665,350 recorded July 9, 2001, at Reel 011964 and Frame 0181.

2. RELATED APPEALS AND INTERFERENCES

The claims pending in the current application are directed to a polypeptide referred to herein as "PRO335." There exists two related patent applications: 1) U.S. Patent Application Serial No. 09/903, 520, filed July 11, 2001 (containing claims directed to PRO335 polypeptides), and 2) U.S. Patent Application Serial No. 09/904,786, filed July 12, 2001 (containing claims directed to PRO335 antibodies). These applications are also under final rejection from the same Examiner and based upon the same type of outstanding rejections, and an appeal of these final rejections is being pursued independently and concurrently herewith.

3. STATUS OF CLAIMS

Claims 1-38, 48 and 53-54 were canceled without prejudice or disclaimer.

Claims 39-47, 49-52 and 55-58 stand rejected in this application and Appellants appeal the rejection of these claims.

4. STATUS OF AMENDMENTS

Claims 39-43 have been amended in a supplemental amendment/response to the Office Action of April 8, 2008 filed concurrently with the present appeal. A copy of the rejected claims in the present Appeal is provided in the Claims Appendix, incorporating the amendment.

5. SUMMARY OF CLAIMED SUBJECT MATTER

The invention claimed in the present application is related to isolated polynucleotides comprising a nucleic acid sequence encoding the polypeptide of SEQ ID NO:290 referred to in the present application as "PRO335," a nucleic acid sequence encoding the polypeptide of SEQ ID NO:290, or a nucleic acid sequence encoding the polypeptide of SEQ ID NO:290 lacking its associated signal peptide; or the full-length coding sequence of the nucleic acid sequence of SEQ ID NO:289; or a nucleic acid sequence of the full-length coding sequence of the cDNA deposited

under ATCC accession number 209927 (Independent Claim 44, and claims 45-47 and 49). The cDNA nucleic acid encoding PRO335 is described in the specification at, for example, page 184, line 21 to page 185, line 32 (Example 43), in Figure 101 and in SEQ ID NO:289. Page 63, lines 34-37 of the specification provides the description for Figures 101 and 102.

The invention is further directed to nucleic acids having at least 80-99% sequence identity to nucleic acids encoding polypeptides of SEQ ID NO:290; or the nucleic acid sequence encoding the polypeptide of SEQ ID NO:290 lacking its associated signal peptide; or the full-length coding sequence of the nucleic acid sequence of SEQ ID NO:289; or the nucleic acid sequence of the cDNA deposited under ATCC accession number 209927, wherein the polypeptide encoded by said nucleic acid is an immunostimulant. PRO nucleic acid variants (Independent Claims 39-43) having at least about 80-99% nucleic acid sequence identity with a nucleic acid encoding for a full length PRO polypeptide sequence or a PRO polypeptide sequence lacking the signal peptide are described in the specification at page 55, line 2 to page 57, line 10, and for example, at page 69, line 25 to page 72, line 8.

The invention is further directed to vectors comprising these nucleic acids and host cells comprising such vectors (page 117 to page 123). The full-length PRO335 polypeptide having the amino acid sequence of SEQ ID NO:290 is described in the specification at, for example, page 50-51, lines 1-22, in Figure 102 and in SEQ ID NO:290. Hybridization probes (Independent Claim 52 and its dependents) and stringent hybridization conditions under which the nucleic acid sequences described above hybridize are described in the specification at, for example, pages 73, line 34 onwards to page 74.

Recombinant expression, characteristics and effects of the PRO335 polypeptides were disclosed in the specification, including in Examples 43, 54, 56, 74, and 77. The PRO335 polypeptides encoded by the claimed nucleic acids were shown to induce proliferation of stimulated T-lymphocytes in a mixed lymphocyte reaction as compared to controls (Example 74). PRO335 is also described as a polypeptide having homology to proteins of the leucine rich repeat superfamily, and particularly, are related to LIG-1 (page 30, line 11, to page 31, line 18, and page 110, lines 26-36). Example 74 (page 208) shows that PRO335 tested positive in the mixed lymphocyte reaction (MLR) assay, demonstrating that PRO335 is active as a stimulator of

the proliferation of stimulated T-lymphocytes, and therefore would have utility in the treatment of conditions where the enhancement of an immune response would be beneficial. In addition, Example 77 shows the ability of PRO335 to stimulate an immune response and induce inflammation at the site of injection in the skin vascular permeability assay, using the hairless guinea pig injected with the Evans blue dye as a model system.

6. GROUNDS OF REJECTION TO BE REVIEWED ON APPEAL

I. Whether the data generated in the MLR assay (Example 74) satisfies the Enablement requirement set forth in 35 U.S.C. § 112, first paragraph, for the invention claimed in Claims 39-47, 49-52 and 55-58.

II. Whether the data generated in the MLR assay (Example 74) satisfies the Written Description requirement set forth in 35 U.S.C. § 112, first paragraph, for the invention claimed in Claims 39-43, 52 and 55-58.

7. ARGUMENT

Summary of the Arguments:

Issue I: Enablement

Appellants submit that patentable utility for the PRO335 polypeptide is based upon data derived from the mixed leukocyte reaction (MLR) assay. The MLR assay is a well-established and accepted assay in the art for evaluating test compounds for their ability to stimulate T-lymphocyte proliferation *in vitro*. Example 74 of the instant specification shows that PRO335 tested positive in the mixed lymphocyte reaction (MLR) assay, demonstrating that PRO335 is active as a stimulator of the proliferation of stimulated T-lymphocytes, and therefore has utility in the treatment of conditions where the enhancement of an immune response would be beneficial, like to treat tumor progression/ regression in cancer. In fact, the Examiner has now acknowledged that the MLR assay is an art accepted assay for identifying immunomodulatory compounds at least on page 3 and on page 11, paragraph 1 of the Final Office Action mailed February 1, 2007 and page 2 of the Office Action mailed April 8, 2008.

The Examiner's primary point to this rejection is that allegedly "the ability of the claimed PRO335 to stimulate or inhibit lymphocyte proliferation in the MLR assay does not provide for what specific conditions or for which specific diseases the claimed invention would predictably function for a therapeutic suppression of the immune system. The assertion that the claimed invention could be useful for the treatment of conditions where the enhancement of the immune response would be beneficial is not enabled by the disclosure of the instant specification." (page 3 of the Office action mailed April 8, 2008). For support, the Examiner quotes Kahan *et al.*, Piccotti *et al.*, and Campo *et al.*, and concludes that "while the art recognizes the MLR assay as accepted for screening for immunosuppressive molecules *in vitro*...this biological activity does not correlate to use of the claimed protein in a therapeutically effective manner, as the asserted use of the claimed invention proposes." (Page 4 of the Office Action mailed April 8, 2008).

Claims 39-47, 49-52 and 55-58 are directed to nucleic acids that encode the polypeptide of SEQ ID NO:290 where the polypeptide has a specific and useful function (*i.e.* as "immunostimulants" useful for boosting the immune system of an animal. Appellants submit that, the instant specification, at least in Example 74, page 208, line 27, and the disclosure of the Fong declaration (submitted with Appellants' response of August 30, 2004), describe the mixed lymphocyte reaction (MLR) assay, which the Examiner has acknowledged as sufficient to establish patentable utility under 35 U.S.C. §101 for the nucleic acids encoding the PRO335 polypeptide. The positive result for PRO335 in the MLR assay demonstrates that PRO335 is active as a stimulator of the proliferation of stimulated T-lymphocytes.

The MLR assay of the instant application is well-described in standard textbooks, including, for example, *Current Protocols in Immunology*, unit 3.12; edited by J.E. Coligan, A.M. Kruisbeek, D.H. Marglies, E.M. Shevach, W. Strober, National Institutes of Health, Published by John Wiley & Sons, Inc. (of record), which is referenced in Example 74. In further support of enablement based upon the MLR assay, the Declaration of Sherman Fong, Ph.D. emphasizes that immunostimulants are important and highly desirable in the treatment of cancer and in enhancing the effectiveness of previously identified treatments for cancer. Costimulation of T cells can induce tumor regression and an antitumor response, both *in vitro* and *in vivo*. In paragraph 9 of his Declaration, Dr. Fong provides examples of important clinical applications for

immune stimulants which have been shown to stimulate T-cell proliferation in the MLR assay. Therefore, based on disclosures in the patent application coupled with information known in the art, one skilled in the art would know that agonistic immunostimulating polypeptides or antibodies are useful in treating, for instance, neoplastic tumors, or antagonistic antibodies – immunosuppressors, are useful for instance, in treating diseases like autoimmune or graft vs. host disease).

Accordingly, Appellants submit that when the proper legal standard is applied, one should reach the conclusion that the present specification provides ample guidance to allow the skilled artisan to make and use those variant nucleic acids that encode for PRO335 polypeptides that are useful in the treatment of conditions like viral infections or cancer, and further, one skilled in the art would know how to use these nucleic acids without any undue experimentation.

Issue II: Written Description

Regarding the written description rejection, Appellants note that the specification provides ample guidance to allow the skilled artisan to identify those nucleic acids with 80-99% identity to the nucleic acid defined in SEQ ID NO.: 289. Further, the Appellants have provided a well-accepted *in vitro* MLR assay can and has been successfully used to identify compounds having immunomodulatory activity *in vivo* (Example 74). Moreover, the instant invention evidences the actual reduction to practice of full-length nucleic acid encoding PRO335 of SEQ ID NO:289, with or without its signal sequence, or encoded by the full-length coding sequence of the cDNA deposited under ATCC accession number 209927.

Appellants further submit that the instant claims are similar to the exemplary claim in Example 10 of the revised Training Manual on Written Description Guidelines issued by the U.S. Patent Office. Appellants respectfully submit that the instant specification evidences the actual reduction to practice of the nucleic acid defined in SEQ ID NO.: 289. Thus, the genus of nucleic acids with at least 80% sequence identity to SEQ ID NO.: 289, would meet the requirement of 35 U.S.C. §112, first paragraph, as providing adequate written description.

These arguments are all discussed in further detail below under the appropriate headings.

Detailed Arguments:

ISSUE I: The Data Generated in the MLR Assay Satisfies the Enablement Requirement of 35 U.S.C. §112, First Paragraph for Claims 39-47, 49-52 and 55-58

Applicants maintain the position that that Claims 39-47, 49-52 and 55-58 satisfy the enablement requirement under 35 U.S.C. §112, first paragraph, for the reasons previously set forth in the Applicants' Responses dated May 17, 2005, November 3, 2006 and July 25, 2007 and Appeal Briefs dated March 10, 2006 and November 27, 2007.

Claims 39-47, 49-52 and 55-58 are directed to a genus of nucleic acid sequences which are at least 80-99% identical to the: nucleic acid encoding the polypeptide of SEQ ID NO:290 or the nucleic acid of SEQ ID NO:289 and which have a specific and useful function (i.e. to the nucleic acids that encode for a genus of polypeptides that are "immunostimulants" useful for boosting the immune system of an animal).

A. Legal Standard for Enablement

According to 35 U.S.C. §112, first paragraph:

The specification shall contain a written description of the invention, and of the manner and process of making and using it, in such full, clear, concise, and exact terms as to enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to make and use the same, and shall set forth the best mode contemplated by the inventor of carrying out his invention.

The test of enablement is whether one skilled in the art could make and use the claimed invention from the disclosure provided by applicants coupled with information known in the art at the time the invention was made, without undue experimentation^{1,2}. Accordingly, the test for enablement is not whether any experimentation is necessary, but whether, if experimentation is

¹ MPEP §2164.0120.

² *United States v. Telecommunications, Inc.* 857.F.2d 778, 785, 8 USPQ2d 1217, 1223 (Fed. Cir. 1998)).

required, it is undue³. The mere fact that an extended period of experimentation is necessary does not make such experimentation undue^{4,5}.

A finding of lack of enablement and a determination that undue experimentation is necessary requires an analysis of a variety of factors (*i.e.*, the *In re Wands* factors). The most important factors that must be considered in this case include 1) the nature of the invention; 2) the level of one of ordinary skill in the art; 3) guidance provided in the specification, 4) the state of the prior art, and 8) the breadth of the claims.

"How a teaching is set forth, by specific example or broad terminology, is not important"^{6,7} "Limitations and examples in the specification do not generally limit what is covered by the claims" MPEP § 2164.08. The test is not merely quantitative, since a considerable amount of experimentation is permissible, if it is merely routine, or if the specification in question provides a reasonable amount of guidance with respect to the direction in which the experimentation should proceed. It is well settled that patent applicants are not required to disclose every species encompassed by their claims, even in an unpredictable art. The legal standard merely requires that there must be sufficient disclosure, either through illustrative examples or terminology, to teach those of ordinary skill how to make and use the invention as broadly as it is claimed⁸.

The M.P.E.P. further states, "The fact that experimentation may be complex does not necessarily make it undue, if the art typically engages in such experimentation. *In re Certain Limited-charge cell Culture Microcarriers*, 221 USPQ 1165, 1174 (Int'l Trade Comm'n 1983), *aff'. sub nom.*, *Massachusetts Institute of Technology v A.B. Fortia*, 774 F.2d 1104, 227 USPQ

³ *In re Angstadt*, 537 F.2d 498,504, 190 USPQ 214, 219 (CCPA 1976).

⁴ *In re Colianni*, 561 F.2d 220,224, 195 USPQ 150, 153 (CCPA 1977).

⁵ MPEP §2164.06.

⁶ MPEP §2164.08.

⁷ *In re Marzocchi*, 439 F. 2d 220,223-4, 169 USPQ 367, 370 (CCPA 1971).

⁸ *Enzo Biochem., Inc. v. Calgene, Inc.*, 188 F.3d 1372 (Fed. Cir. 1999) (quoting *In re Vaeck*, 947 F.2d 488,496 (Fed. Cir. 1991)).

428 (Fed. Cir. 1985) M.P.E.P. §2164.01. A considerable amount of experimentation is permissible, if it is merely routine.

B. Proper Application of the Legal Standard

Initially, Applicants submit that, both, the instant specification (in Example 74) and the Fong declaration (in previously submitted Exhibit A of the declaration) clearly refer to and incorporate by reference contents of the book "Current Protocols in Immunology, unit 3.12; edited by JE Coligan, AM Kruisbeek, DR Margulies, EM Shevach, W Stober, National Institutes of Health, Published by John Wiley & Sons, Inc. (1991) (referred to henceforth as "Current protocols"). "Current protocols" provides the detailed basic protocol, for instance, at least in Unit 3.12.6 entitled "T cell proliferation in mixed lymphocyte cultures" and further provides various other protocols for measuring T lymphocyte activation. It also provides methods for preparing cells and materials useful in the T lymphocyte activation assays and teaches that an MLR reaction can be monitored qualitatively, for example, by following the incorporation of tritiated thymidine during DNA synthesis, or, by observing blast formation, or by other methods well known in the art. Applicants submit that this information was readily available at the time of filing of the application, since the "Current protocols" reference was disclosed and incorporated by reference in its entirety at the time of filing.

Further, Applicants have provided native PRO sequence SEQ ID NO: 290. The specification also describes methods for the determination of percent identity between two amino acid sequences. In fact, the specification teaches specific parameters to be associated with the term "percent identity" as applied to the present invention. Accordingly, one of skill in the art could identify whether the variant PR0335 native sequence falls within the parameters of the claimed invention. Once such an amino acid sequence was identified, the specifications sets forth methods for making the amino acid sequences and methods of preparing the PRO polypeptides. Accordingly, one skilled in the art given the disclosure in the specification would be able to make the claimed amino acid sequence. Furthermore, one of ordinary skill in the art has a sufficiently high level of technical competence to identify sequences with at least 80%

identity to SEQ ID NO: 290. Accordingly, one of ordinary skill could make the claimed invention without undue experimentation.

The Examiner's primary point to this rejection is that allegedly "the ability of the claimed PRO335 to stimulate or inhibit lymphocyte proliferation in the MLR assay does not provide for what specific conditions or for which specific diseases the claimed invention would predictably function for a therapeutic suppression of the immune system. The assertion that the claimed invention could be useful for the treatment of conditions where the enhancement of the immune response would be beneficial is not enabled by the disclosure of the instant specification." (page 3 of the Office action mailed April 8, 2008). For support, the Examiner quotes Kahan *et al.*, Piccotti *et al.*, and Campo *et al.*, and concludes that "while the art recognizes the MLR assay as accepted for screening for immunosuppressive molecules *in vitro*...this biological activity does not correlate to use of the claimed protein in a therapeutically effective manner, as the asserted use of the claimed invention proposes." (Page 4 of the Office Action mailed April 8, 2008).

Claims 39-47,49-52 and 55-58 are directed to antibodies to the polypeptide of SEQ ID NO:290 where the polypeptide has a specific and useful function (*i.e.* as "immunostimulants" useful for boosting the immune system of an animal. Applicants submit that, the instant specification, at least in Example 74, page 208, line 27, and the disclosure of the Fong declaration (submitted with Applicants' response of August 30, 2004), describe the mixed lymphocyte reaction (MLR) assay, which the Examiner has acknowledged as sufficient to establish patentable utility under 35 U.S.C. §101 for the nucleic acids encoding the PRO335 polypeptide. The positive result for PRO335 in the MLR assay demonstrates that PRO335 is active as a stimulator of the proliferation of stimulated T-lymphocytes. Therefore, based on disclosures in the patent application coupled with information known in the art, one skilled in the art would know that agonistic immunostimulating polypeptides and/or antibodies are useful in treating, for instance, neoplastic tumors, or antagonistic antibodies –immunosuppressors, are useful for instance, in treating diseases like autoimmune or graft vs. host disease).

The MLR assay of the instant application is well-described in standard textbooks, including, for example, *Current Protocols in Immunology*, unit 3.12; edited by J.E. Coligan,

A.M. Kruisbeek, D.H. Marglies, E.M. Shevach, W. Strober, National Institutes of Health, Published by John Wiley & Sons, Inc. (of record), which is referenced in Example 74.

In further support of utility based upon the MLR assay, Applicants have submitted (with their Response filed August 30, 2004) the Declaration of Sherman Fong, Ph.D. As Dr. Fong emphasizes, immunostimulants are important and highly desirable in the treatment of cancer and in enhancing the effectiveness of previously identified treatments for cancer. Costimulation of T cells can induce tumor regression and an antitumor response, both *in vitro* and *in vivo*. In paragraph 9 of his Declaration, Dr. Fong provides examples of important clinical applications for immune stimulants which have been shown to stimulate T-cell proliferation in the MLR assay. As Dr. Fong explains,

IL-12 is a known immune stimulant, which has been shown to stimulate T-cell proliferation in the MLR assay. IL-12 was first identified in just such an MLR [Gubler et al. PNAS 88, 4143 (1991) (Exhibit C)]. In a recent cancer vaccine trial, researchers from the University of Chicago and Genetics Institute (Cambridge, MA) have demonstrated the efficacy of the approach, relying on the immune stimulatory activity of IL-12, for the treatment of melanoma. [Peterson et al. Journal of Clinical Oncology 21 (12). 2342-48 (2003) (Exhibit D)]

Dr. Fong concludes that (paragraph 10):

It is my considered scientific opinion that a PRO polypeptide shown to stimulate T-cell proliferation in the MLR assay of the present invention with an activity at least 180% of the control, as specified in the present application, is expected to have the type of activity as that exhibited by IL-12, and would therefore find practical utility as an immune stimulant."

Therefore, one skilled in the art would know that immunostimulating compounds like IL-12 or PRO335 of this invention, could be used in immunoadjuvant therapy (with tumor-specific antibodies) for the treatment of tumors (cancer) and could be administered alone or together with other agents to stimulate T cell proliferation/ activation (immune function). Accordingly, the positive results obtained in this assay clearly establish the immunostimulant utility for the PRO335 polypeptides and its encoding nucleic acids claimed in the present application, and the specification, in turn, enables one skilled in the art to use the compounds for the asserted purpose.

Applicants further submit that the MLR assay was routinely used in the art to identify immunostimulants or immunosuppressors, and additionally, were found to have *in vivo* utility, in the treatment of various diseases and conditions. Applicants incorporate by reference the articles and arguments presented in the Response filed November 3, 2006 (see Santoli *et al.*, J. Immunol. 137:400-407 (1986); U.S. Patent Application No. 4,950,647, Reddy *et al.* (Infect. Immun. 44:339-343 (1984); Pahwa *et al.* (Proc. Natl. Acad. Sci. USA 86:5069-5073 (1989); Kirchner *et al.* (Br. J. Clin. Pharmacol. 46:5-10 (1998); Grabstein, K.H. *et al.*, Science 264:965-968 (1994); Chapoval *et al.* (J. Immunol. 161:6977-6984 (1998); Kasaian, M.T. *et al.*, Immunity 16:559-569 (2002); Ma *et al.* (J. Immunol. 171:608-615 (2003); Naito, K. *et al.*, J. Immunol. 142:1834-1839 (1989); Tarr, P.E, Med. Oncol. 13:133-140 (1996); Gennari *et al.* (Annals of Surgery, 220:68-76 (1994); Patterson, S. *et al.*, J. Immunol. 175:5087-5094 (2005); Toura *et al.* (J. Immunol. 163:2387-2391 (1999); Tsavaris *et al.*, Br. J. Cancer 87:21-27 (2002); Amirghofran, Z. *et al.*, Ivn. J. Med. Sci. 25:119-124, (2000); Abolhassani, M., Brazilian Journal of Infectious Diseases 8:382-385, (2004); U.S. Patent No. 5,817,306, filed June 7, 1995; U.S. Patent No. 5,801,193, filed April 15, 1997; U.S. Patent No. 5,958,403, filed July 11, 1994 ; and U.S. Patent No. 5,648,376, filed January 19, 1995.

Applicants further note that a positive result as a stimulator in the MLR assay is also characteristic of molecules which have known *in vivo* utilities in the treatment of disorders for which stimulation of an immune response is desirable. For example, as discussed above IL-12 is a known immune stimulant, which has been shown to stimulate T-cell proliferation in the MLR assay (Gubler *et al.*, PNAS 88:4143 (1991) (submitted as Exhibit C in Applicants' Response filed August 30, 2004). In a recent cancer vaccine trial, researchers from the University of Chicago and Genetics Institute (Cambridge, MA) have demonstrated the efficacy of an approach relying on the immune stimulatory activity of IL-12 for the treatment of melanoma. Peterson *et al.*, J. Clin. Oncol. 21:2342-2348 (2003) (submitted as Exhibit D in Applicants' Response filed August 30, 2004).

Thus, the art as a whole, at the time of filing of the application, clearly establishes that the mixed lymphocyte reaction (MLR) is a widely used *in vitro* assay for identifying immunomostimulatory compounds and that the positive result as a stimulator in the MLR assay

is widely accepted as a valid indication of therapeutic use in the treatment of disease conditions, including irradiation of tumors. Applicants note that Dr. Fong's conclusions are consistent with what is accepted in the art. Accordingly, one skilled in the art would know how to use the compounds for the asserted purpose. Therefore, based on the art's teachings about the immunostimulatory activity of molecules, as a result of a positive MLR assay, would provide sufficient correlation to one skilled in the art, such that they would use the identified compounds in the treatment of disorders for which stimulation of the immune system is beneficial, such as viral or bacterial infections, immune deficiencies, or tumor/cancer treatments.

The Examiner asserts that "the conclusions reached by Fung-Leung et al. are based on much more experimental data, assays and testing than that provided in the instant specification and the reference does not support the position that the MLR assay in the instant specification is predictive of use as a therapeutic compound for suppressing the immune response.." (Page 6 of the Office Action mailed April 8, 2008).

Applicants submit that Applicants need not disclose every teaching found in the post-filing references. Indeed, the present specification teaches enabling disclosure for the claimed invention and the post-filing references merely confirm the feasibility of the present invention as disclosed in the specification. The pre- and post filing published papers submitted by Applicants were intended to demonstrate the MLR assay was routinely used in the art to identify immunostimulants or immunosuppressors, and additionally, were found to have *in vivo* utility, in the treatment of various diseases and conditions. In addition to the specific disclosure in the specification, general knowledge in the art at the time the invention was made also must be taken into account when assessing compliance with the enablement requirement of 35 U.S.C. §112, first paragraph. Based on the art's teachings about the immunostimulatory activity of molecules, a result of a positive MLR assay would provide sufficient correlation to one skilled in the art, such that they would use the identified compounds in the treatment of disorders for which stimulation of the immune system is beneficial.

The Examiner asserts that Steinman and Thurner "address the utility of dendritic cells but not of a stimulatory MLR." (Page 7 of the Office Action mailed April 8, 2008).

Applicants submit that, as indicated in Unit 3.12.9 of Current Protocols in Immunology, dendritic cells are stimulator lymphocytes that induce responder T cells and activate them to increase cytokine production, cytokine receptor expression, and ultimately proliferation of the activated T cells, all of which are measurable in different assays. In the current MLR assay, suspensions of responder T cells were cultured with irradiated- or mitomycin treated- allogenic stimulator lymphocytes and thymidine uptake was measured to give a measure of T cell proliferation (see Current protocols, Unit 3.12.9). Current Protocols also teaches how stimulator lymphocytes (which includes dendritic cells) induce responder T cells and methods of preparing them. Thus, based on this disclosure, one skilled in the art would know how to use dendritic cells in an MLR assay and how to measure T lymphocyte stimulation using thymidine uptake.

Regarding the rejection based on the Gubler reference, the Examiner alleges that it "describes the identification of IL-12 but uses MLR merely to compare activities, not as the basis for describing a molecule as a therapeutically useful immunostimulant." (Page 7 of the Office Action mailed April 8, 2008)

Applicants respectfully disagree. The use of the MLR assay has been extensively reviewed above under utility. Several peer-reviewed references and issued patents acknowledge its usefulness (see above, utility Section I). Applicants add that in fact, the Gubler reference clearly teaches the MLR assay (see the footnote of Table 1, Fig. 3(upper panel) and related discussions in the results section), where PHA-activated lymphoblasts prepared from human PBMCs were used to measure lymphoblast proliferation in a tritiated thymidine assay. This assay was a key assay in identifying IL-12 as an immunostimulant for T lymphocytes with immunoenhancing effects. Again, this is evidenced since Gubler discloses in column 1, page 4143 that "we initiated a search for novel cytokines that would synergize with suboptimal concentrations of recombinant IL-2 to activate cytotoxic lymphocytes *in vitro* and thus might have synergistic immunoenhancing effects when administered together with recombinant IL-2 *in vivo*" (emphasis added). Thus the Gubler reference also supports the Applicants position that the MLR assay is very useful in identifying immunostimulants.

Regarding the rejection based on the Peterson reference and the use of IL-12 as an immunostimulant, the Examiner says that Peterson's subsequent research "was clearly required to suggest that the molecule could be used in this fashion". (Page 7 of the Office Action mailed April 8, 2008)

Again, Applicants respectfully disagree. Even though the Peterson's reference was published after the effective filing date of the instant application, it is an enabling reference, and its teachings are not contrary to the teachings of other references found in the art at, or before the time of filing of the instant application (July 18, 2001). For instance, Toura *et al.* (J. Immunol. 163:2387-2391 (1999); of record) disclosed that the "[i]njection of α -GalCer inhibits tumor metastasis almost completely in the liver or lung" (page 2387, col. 2). Toura *et al.* found that dendritic cells pulsed with α -GalCer are able to induce antitumor activity *in vivo* within 24 hours after cell transfer (page 2390, col. 2). Chapoval *et al.* (J. Immunol. 161:6977-6984 (1998); of record) further studied the impact of IL-15 as an adjuvant to cancer therapy using cyclophosphamide (CY) in a mouse lung tumor model. GM-CSF is used in cancer immunotherapy to expand the population of dendritic cells before reinfusion into the patient (page 136, col. 2; Tarr, P.E, Med. Oncol. 13:133-140 (1996); of record). Kirchner *et al.* (Br. J. Clin. Pharmacol. 46:5-10 (1998); of record) stated that "[t]he use of recombinant human interleukin-2 (rhIL-2) has been recommended as the best current therapy for advanced renal cell carcinoma" (page 5, col. 1). Santoli *et al.*, J. Immunol. 137:400-407 (1986); of record), and in U.S. Patent Application No. 4,950,647 (column 9, lines 30-36; Table III; of record). Based upon its immunostimulatory activity, IL-2 has been demonstrated to have a range of utilities in the treatment of immune deficiencies, as well as in immunotherapy for cancer.

The Peterson reference further supports the use of the immunostimulant IL-12 in the treatment of a cancer, namely, melanoma. As exemplified from the list of references discussed above, the use of immunostimulants in the treatment of cancer was not concluded based on the Peterson studies alone. In fact, Gubler *et al.* (discussed in the Fong Declaration) also indicates on column 1, page 4143, that "we initiated a search for novel cytokines that would synergize with suboptimal concentrations of recombinant IL-2 to activate cytotoxic lymphocytes *in vitro* and thus might have synergistic immunoenhancing effects when administered together with

recombinant IL-2 *in vivo*" (emphasis added). Therefore, Peterson *et al.* is in fact a supportive and enabling reference, indicating the use of immunostimulant molecules in the successful treatment of cancer.

*The Examiner cites the reference Kahan (1991) for its statement that "no in vitro assay predicts or correlates with in vivo immunosuppressive efficacy; there is no surrogate immune parameter as a basis of immunosuppressive efficacy and/or for dose extrapolation from in vitro systems to in vivo conditions," (page 3 of the instant Office action). The Examiner further cites Piccotti *et al.* (1999) to show that "IL-12 enhances alloantigen-specific immune function as determined by MLC, but this result in vitro does not result in a measurable response in vivo" (page 3 of the instant Office action). The Examiner further cites Campo *et al.* (2001) and says "while zinc suppresses alloreactivity in MLC, it does not decrease T-cell proliferation in vitro nor produce immunosuppressive effects in vivo", (pages 3-4 of the Office Action mailed April 8, 2008).*

Applicants respectfully disagree. Applicants submit that the Examiner has not correctly characterized the teachings of Kahan *et al.*, Piccotti *et al.* and Campo *et al.* On the other hand, these references, in combination with those cited by Applicants, demonstrate that, the art as a whole recognizes that the mixed lymphocyte reaction (MLR) is a widely used *in vitro* assay for identifying immunomodulatory compounds.

For instance, the statement by Kahan *et al.* (see above) is inconsistent with what was known and accepted in the art at the time of filing regarding the MLR assay. For example, U.S. Patent No. 5,817,306 states, "The mixed lymphocyte response (MLR) and phytohemagglutinin A (PHA) assays are valuable for identifying immune suppressive molecules *in vitro* that are useful for treating graft versus host disease. **The results obtained from these assays are generally predictive of their *in vivo* effectiveness.**" (Column 12, lines 36-41; emphasis added). U.S. Patent No. 5,801,193, filed April 15, 1997, states that "**[t]he MLR is an assay recognized by those skilled in the art as an in vitro predictor of *in vivo* immunosuppressant activity.**" (Column 8, lines 8-10, emphasis added). U.S. Patent No. 5,648,376, filed January 19, 1995, states that "[a] measure of immunosuppression that serves as a model for transplantation

rejection is inhibition of cell proliferation in a mixed lymphocyte reaction (MLR) assay.” (Column 11, lines 24-26). Therefore, Kahan's quoted statement contradicts well established scientific wisdom. As discussed extensively above, in fact, the MLR assay has been extensively used and is the best *in vitro* model for screening immunostimulatory agents. In fact, the examiner's cited reference, Picotti *et al.*, also supports this point, since the authors extensively used the MLC assay in their studies.

Picotti *et al.* studied the mechanism of alloimmune response and graft rejections. Picotti *et al.* in fact, confirms that “IL-12 is a key cytokine involved in promoting cell mediated immune responses *in vivo*” (page 1459, col. 1). Picotti *et al.* also showed that the IL-12R gamma subunit was critical for IL-12 driven enhanced alloimmune response *in vitro* and *in vivo* (see abstract). Based on their studies, one skilled in the art would know that immunostimulating compounds like IL-12 (or of this invention) could be used in immunoadjuvant therapy (with tumor-specific antibodies, which is also discussed in the Fong Declaration, Petersen *et al.* reference) for the treatment of tumors (cancer). One skilled in the art would know that immunostimulant molecules can be administered alone or together with other agents to stimulate T cell proliferation/ activation (immune function) ad therefore, one skilled in the art would know that such agents can be used to stimulate an antitumor response to a tumor antigen. If anything, Picotti *et al.*, supports the point that immunostimulants are useful for treating tumors.

Applicants respectfully point out that the Examiner has misinterpreted this statement, due to the fact that the authors refer to two different types of immunosuppressive effects. Campo *et al.* set out to look for an inhibitor of MHC *in vitro* which would have the fewest side effects *in vivo* (see Abstract). The authors note that high concentrations of zinc “impair **all** T cell and monocyte function” (page 20; emphasis added). The authors took this impairment as an indicator of toxicity, and therefore intentionally used concentrations of zinc below that at which all T-cell function was impaired, in order to identify a concentration range that would not result in toxic effects. However, that does not mean that Campo *et al.* found zinc to have no immunosuppressive activity *in vivo*. In fact, the authors conclude, based upon their MLC results, that “zinc could become an immunosuppressant in transplantation medicine without toxic side effects” (page 21; emphasis added). Thus Campo *et al.* supports Applicants' position that

those of skill in the art would interpret the results of MLC assays as having physiological relevance.

Applicants note that the Examiner has failed to point out several instances within these cited references wherein the authors stated that the MLR is an important method with a good predictive value. For example, Campo *et al.* teach that “the human mixed lymphocyte culture (MLC) is an important method to test donor-recipient compatibility in bone marrow transplantation. It could be shown that cytokine release, especially IFN-gamma, **has a very good predictive value with regard to the transplantation outcome**, as cytokines play a major role in the generation of an alloreactive immune response and for the induction of graft rejection *in vivo*.....Landolfo *et al.* inhibited T-cell reactivity by the addition of anti-IFN-gamma **both *in vitro* and *in vivo***” (see page 18; emphasis added). Finally, Campo *et al.* teaches that “cyclosporin A, FK506, and other substances are used to prevent graft rejection. **In vitro experiments revealed an inhibition of the MLC**” (page 16). Thus the teachings of Campo *et al.* confirm that inhibition of the MLR is observed for known immunoinhibitory molecules, that are in actual clinical use.

Thus, while there are instances of unpredictability in some studies using the MLR assay, there are many more studies showing the usefulness and predictable results using MLR, as exemplified by the studies by Picotti, Landolfo and the IFN-gamma study and all the references submitted by the Applicants in this response. Therefore, the teachings within Kahan *et al.*, Piccotti *et al.*, Campo *et al.*, in fact, support the usefulness of the MLR assay.

The Examiner further asserts that “the results of the MLR assay in the instant specification are merely preliminary, and much more experimentation is necessary for one of ordinary skill in the art to use the claimed invention in the manner disclosed.” (Page 6 of the Office Action mailed April 8, 2008)

Applicants respectfully submit that enablement “is not precluded even if some experimentation is necessary, although the amount of experimentation needed must not be unduly extensive.” As the M.P.E.P. states, “[t]he fact that experimentation may be complex does

not necessarily make it undue, if the art typically engages in such experimentation.” The M.P.E.P. further explains that “If a statement of utility in the specification contains within it a connotation of how to use, and/or the art recognizes that standard modes of administration are known and contemplated, 35 U.S.C. 112 is satisfied.” Applicants note that the specification clearly indicates that the claimed polypeptides are useful in the treatment of undesirable immune responses. The use of immunosuppressive molecules in the treatment of such disorders is well known in the art, as indicated by Kahan *et al.*, Picotti *et al.* and Campo *et al.*, made of record by the Examiner, as well as the references and U.S. Patents, previously discussed and made of record by Applicants. Thus any further experimentation required for determining, for example, a particular dosage or method for the administration of PRO335 would not be considered undue.

Further, with respect to disclosure of the results of *in vitro* assays, the M.P.E.P. states that “if the art is such that a particular model is recognized as correlating to a specific condition, then it should be accepted as correlating unless the examiner has evidence that the model does not correlate. Even with such evidence, the examiner must weigh the evidence for and against correlation and decide whether one skilled in the art would accept the model as reasonably correlating to the condition. *In re Brana*, 51 F.3d 1560, 1566, 34 U.S.P.Q.2d 1436, 1441 (Fed. Cir. 1995).”

The M.P.E.P. also makes it clear that the burden of proof is on the Examiner, to demonstrate lack of correlation for an *in vitro* model. “(s)ince the initial burden is on the examiner to give reasons for the lack of enablement, the examiner must also give reasons for a conclusion of lack of correlation for an *in vitro* or *in vivo* animal model example.” A rigorous or an invariable exact correlation is not required, as stated in *Cross v. Iizuka*, wherein the court stated that “based upon the relevant evidence as a whole, there is a reasonable correlation

between the disclosed *in vitro* utility and an *in vivo* activity, and therefore a rigorous correlation is not necessary where the disclosure of pharmacological activity is reasonable based upon the probative evidence.”

As discussed above, MLR was routinely used in the art to identify immunostimulants or immunosuppressors, and additionally, were found to have *in vivo* utility, in the treatment of various diseases and conditions. Further, the importance of immunostimulants in the treatment of cancer or in enhancing the effectiveness of previously identified treatments for cancer, including tumor-specific antibodies were well known in the art at the time of filing of the instant application, as discussed in several references cited above. For instance, costimulation of T cells inducing tumor regression and an antitumor response, both *in vitro* and *in vivo* was known (for e.g., Steinman *et al.* -submitted as Exhibit B with the Response filed August 30, 2004). Thus, one skilled in the art would know that immunostimulating compounds like IL-12 or PRO335 of this invention, could be useful in immunoadjuvant therapies, for the treatment of tumors (cancer) and could be administered either alone or together with other agents to stimulate T cell proliferation/ activation (immune function). These could be done without undue experimentation.

*The Examiner asserts that “Current Protocols in Immunology in fact describes many variables that must be controlled for. In the instant application, no such controls, such as for maximum response or for the inherent variability of individual responses, are provided. There is no indication of statistical significance of the results. There are no autologous controls. No correlation is provided to any particular *in vivo* function; there is no guidance to indicate that PRO335 could be used to any therapeutic effect for the treatment of diseases such as cancer or HIV.” (Page 7 of the Office Action mailed April 8, 2008)*

Applicants respectfully maintain their position, as presented in the Response filed November 3, 2006, that the controls cited by the Examiner were only needed for the purpose of evaluating the properties of the stimulator cells. Such determinations, however, are not required for the MLR assay of Example 74, and thus these controls are not “essential”. Because the

response in the test reaction is compared to a negative control reaction, and because both reactions use the same stimulator and responder cells at the same time, additional controls to determine the precise properties of these cells are not required. Further, the protocols described in the instant specification are consistent with those accepted in the art. For example, U.S. Patent No. 4,950,647, which demonstrated the immunoenhancing activity of the compound 6-Amino(2-deoxy-alpha-D-erythro-petofuranosyl)imidazo[4,5,-C] pyridine-4-one using the MLR assay, did not disclose the use of any additional controls beyond those disclosed in the instant application.

With respect to the statistical significance of the results, Applicants respectfully submit that these remarks are a clear indication that the Examiner applies a heightened legal standard that is inappropriate for determining if the “enablement” standard of the Patent Statute is met. First of all, as evidenced in the numerous references made of record by Applicants, knowledge in the art at the time the invention was filed clearly demonstrate an ability to determine statistical significance of results generated from the MLR assay. Further, the MLR assay described herein is a comparative one (increases of greater than or equal to 180% is preferred), meaning that the utility is based upon a comparison of relative expression levels between a known polypeptide and an unknown PRO molecule. Useful information is obtained when a relative differences are observed, and this is routine in biological testing. Applicants expressly assert that the observed difference for PRO335 is significant (this point is further discussed below based on U.S. Patent No. 4,950,647). For instance, Example 74 of the specification makes clear the standard to be used to determine whether a positive result in the MLR assay is significant, stating that “[p]ositive increases over control in this assay are considered to be positive results, with increases of greater than or equal to 180% being preferred and that PRO335 tested positive in this assay. However, any value greater than control indicates a stimulatory effect for the test protein” (page 203, line 27). Therefore, this disclosure clearly meets the standard for statistical significance. The Examiner seems to focus on exactly how much higher (*i.e.*, requiring Applicants to provide “relative or absolute levels” and statistical analyses), but Applicants submit that this is not relevant to the issue at hand, nor is it required for the claimed invention to be useful.

Applicants further submit that the term “positive increases over control” would readily be understood by one skilled in the art. For instance, the Examiner’s attention is directed to U.S. Patent No. 4,950,647 (of record), which claims immunoenhancing compositions comprising the compound 6-Amino(2-deoxy-alpha-D-erythro-petofuranosyl)imidazo[4,5,-C] pyridine-4-one. The immunoenhancing activity of the claimed compound was determined in part by the use of the MLR assay, as shown in Example IV (column 13, lines 20-37). The claimed compound increased the response in the MLR, with a maximum increase of 191% as compared to control, as shown in Table VII. IL-2 showed a similar level of stimulation of the MLR (with a maximum of 200% as compared to control) as shown in Table III. Thus this patent supports the threshold of 180% described in the instant specification as showing significant stimulatory activity. Given that 6-Amino(2-deoxy-alpha-D-erythro-petofuranosyl)imidazo[4,5,-C] pyridine-4-one was identified as an immunostimulatory compound based upon a reported increase in the MLR assay of 191% as compared to control, the activity for PRO335 of greater than or equal to 180% as compared to control clearly meets the standard accepted in the art as demonstrating patentable utility.

Therefore, this rejection requiring allegedly essential controls and statistical data are not appropriate, as relevant even from the art, and should be withdrawn.

As set forth in M.P.E.P, 2107 II(B)(I), if the applicant has asserted that the claimed invention is useful for any particular practical purpose, and the assertion would be considered credible by a person of ordinary skill in the art, a rejection based on lack of utility should not be imposed. The logic underlying the asserted utilities in the present case is not inconsistent with general knowledge in the art, and would be considered credible by a person skilled in the art. It is, of course, always possible that an invention fails on its way of development to a commercial product. Thus, despite recent advances in rational drug design, a large percentage of drug candidates fails, and never makes it into a drug product. However, the USPTO is not the FDA, the law does not require that a product (drug or diagnostic) be currently available to the public in order to satisfy the utility requirement.

Further, the test of enablement is whether one reasonably skilled in the art could make or use the invention from disclosures in the patent application coupled with information known in the art without undue experimentation. *United States v. Teletronics, Inc.*, 857F.2d 778, 785 (Fed. Cir. 1988), Emphasis added. Thus, in addition to the specific disclosure in the specification, general knowledge in the art at the time the invention was made also must be taken into account when assessing compliance with the enablement requirement of 35 U.S.C. §112, first paragraph. The full-length PRO335 polypeptide having the amino acid sequence of SEQ ID NO:290 is described in the instant specification at, for example, page 50-51, lines 1-22, in Figure 102 and in SEQ ID NO:290. Support for the preparation and uses of nucleic acids is found throughout the specification, including, for example pages 55-57 and 117-123.

Applicants respectfully remind the Examiner that the skilled artisan in the field of Immunology and Immunotherapeutics, at the effective filing date of November 12, 1997, would likely be a person with a Ph. D. or M.D. degree, sometimes both, with extensive experience. As such, one skilled in the art could easily test whether the PRO335 polypeptides encoded by the claimed nucleic acids can enhance T-cell stimulatory activity using the MLR assay (as described in the Example 74 of the specification and in Current Protocols). As the M.P.E.P. states, "[t]he fact that experimentation may be complex does not necessarily make it undue, if the art typically engages in such experimentation." M.P.E.P. §2164.01 Thus, one would have known how to make and use the present invention at the effective date of the application.

In summary, in view of the foregoing arguments, the examples and specific teachings provided in the specification and general knowledge in the art, one skilled in the art at the priority date of the present application would have clearly known how to use the invention within the full scope of the claims pending. Accordingly, Appellants respectfully request reconsideration and reversal of the enablement rejection of Claims 39-47, 49-52 and 55-58 under 35 U.S.C. §112, first paragraph.

ISSUE II: The Data Generated in the MLR Assay Satisfies the Written Description Requirement of 35 U.S.C. § 112, First Paragraph for Claims 39-43, 52 and 55-58

Claims 39-43, 52 and 55-58 are rejected under 35 U.S.C. §112, first paragraph, allegedly because the specification does not describe the claimed invention in such a way as to reasonably convey to one skilled in the art that the inventors, at the time the application was filed, had possession of the claims invention. (Page 9 of the Office Action mailed April 8, 2008).

In particular, the Examiner has taken the position that, while the specification provides adequate description for the nucleic acid encoding the polypeptide of SEQ ID NO:290, there is insufficient written description as to the identity of a nucleic acids encoding polypeptides having at least 80% to 99% sequence identity to SEQ ID NO:290. The Examiner has asserted that the nucleic acids encoding the PRO335 polypeptide as encompassed with the broad definition of 80% to 99% identical to SEQ ID NO:290 are all required to practice the instantly claimed invention, and as stated in the previous office action, the specification does not provide an adequate written description of the broad genus having potentially highly diverse functions as encompassed by the phrase 80% to 99% sequence identity.

Coupled with the general knowledge available in the art at the time of the invention, Appellants submit that the specification provides ample written support for the claimed polypeptides. Thus, based on the high percentage of sequence identity, one skilled in the art would have known at the time of the invention that the Appellants had possession of the claimed polypeptides.

A. The Legal Test for Written Description

The well-established test for sufficiency of support under the written description requirement of 35 U.S.C. §112, first paragraph is “whether the disclosure of the application as originally filed reasonably conveys to the artisan that the inventor had possession at that time of the later claimed subject matter, rather than the presence or absence of literal support in the specification for the claim language.”^{12, 13} The adequacy of written description support is a factual issue and is to be determined on a case-by-case basis.¹⁴ The factual determination in a

¹² *In re Kaslow*, 707 F.2d 1366, 1374, 212 U.S.P.Q. 1089, 1096 (Fed. Cir. 1983).

¹³ See also *Vas-Cath, Inc. v. Mahurkar*, 935 F.2d at 1563, 19 U.S.P.Q.2d at 1116 (Fed. Cir. 1991).

¹⁴ See e.g., *Vas-Cath*, 935 F.2d at 1563; 19 U.S.P.Q.2d at 1116.

written description analysis depends on the nature of the invention and the amount of knowledge imparted to those skilled in the art by the disclosure.^{15, 16}

In *Environmental Designs, Ltd. v. Union Oil Co.*,¹⁷ the Federal Circuit held, “Factors that may be considered in determining level of ordinary skill in the art include (1) the educational level of the inventor; (2) type of problems encountered in the art; (3) prior art solutions to those problems; (4) rapidity with which innovations are made; (5) sophistication of the technology; and (6) educational level of active workers in the field.” (Emphasis added).¹⁸ Further, The “hypothetical ‘person having ordinary skill in the art’ to which the claimed subject matter pertains would, of necessity have the capability of understanding the scientific and engineering principles applicable to the pertinent art.^{19, 20}

B. The Disclosure Provides Sufficient Written Description for the Claimed Invention

Appellants respectfully submit that the instant specification evidences the actual reduction to practice of the amino acid sequence of SEQ ID NO:290. Thus, the genus of nucleic acids encoding polypeptides with at least 80% sequence identity to SEQ ID NO:290, would meet the requirement of 35 U.S.C. §112, first paragraph, as providing adequate written description.

Appellants respectfully submit that the instant claims are similar to the exemplary claim in Example 10 of the revised Training Manual on Written Description Guidelines issued by the U.S. Patent Office.

Example 10 of the Training Manual clearly states that the protein variants meet the requirements of 35 U.S.C. §112, first paragraph, as providing adequate written description for

¹⁵ *Union Oil v. Atlantic Richfield Co.*, 208 F.2d 989, 996 (Fed. Cir. 2000).

¹⁶ See also M.P.E.P. §2163 II(A).

¹⁷ 713 F.2d 693, 696, 218 U.S.P.Q. 865, 868 (Fed. Cir. 1983), cert. denied, 464 U.S. 1043 (1984).

¹⁸ See also M.P.E.P. §2141.03.

¹⁹ *Ex parte Hiyamizu*, 10 U.S.P.Q.2d 1393, 1394 (Bd. Pat. App. & Inter. 1988) (emphasis added).

²⁰ See also M.P.E.P. §2141.03.

the claimed invention even if the specification contemplates but does not exemplify variants of the protein if: (1) the procedures for making such variant proteins is routine in the art, (2) the specification does not describe the complete structure or physical properties of the variants, although those skilled in the art would expect members of the genus to have properties similar to those of the reference sequence because of high degree of structural similarity, and (3) the variant proteins of the genus possess a significant degree of partial structure (see Claim 2 of Example 10).

Appellants submit that all the requirements in Example 10 are met for the variant nucleic acids encoding PRO335 polypeptides of the instant claims . In particular, Claims 39-43 require that the variant nucleic acids encoding the polypeptide of PRO335 share a high sequence identity to SEQ ID NO:290. In addition, the procedures of making variant nucleic acid encoding the polypeptide of SEQ ID NO:290 are well-known in the art and described in detail in the specification. The instant specification includes extensive step-by-step guidance in the specification on how to make and prepare nucleic acids where the endcoded polypeptides have 80% to 99% identity to the polypeptide of SEQ ID NO: 290. For instance, the specification describes methods for the determination of percent identity between two sequences. In fact, the specification teaches specific parameters to be associated with the term "percent identity" as applied to the present invention. The specification further provides detailed guidance as to changes that may be made to a PRO polypeptide without adversely affecting its activity. This guidance includes a listing of exemplary and preferred substitutions for each of the twenty naturally occurring amino acids (Table 6). Accordingly, one of skill in the art could identify whether a variant PRO335 sequence falls within the parameters of the claimed invention. Once such a nucleic acid sequence is identified, the specification sets forth methods for making and preparing the PRO polypeptides. Appellants claim only those nucleic acids encoding polypeptides which meet the stated guidelines.

Therefore, Appellants submit that the specification provides ample guidance such that one of skilled in the art would know that Appellants possessed the invention as claimed in the instant claims, at the time of filing of the application. Accordingly, Appellants respectfully request reconsideration and reversal of this outstanding rejection under 35 U.S.C. §112, first

paragraph. Accordingly, Appellants respectfully request reconsideration and reversal of the written description rejection of Claims 39-43, 52 and 55-58 under 35 U.S.C. §112, first paragraph.

CONCLUSION

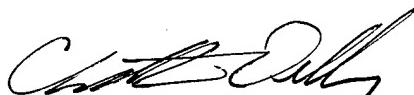
For the reasons given above, Appellants submit that the MLR assay disclosed in Example 74 of the specification provides at least one patentable utility for the PRO335 polypeptides of Claims 39-47, 49-52 and 55-58. In addition, Claims 39-47, 49-52 and 55-58 meet the requirements of 35 U.S.C. §112, first paragraph - enablement and written description. Accordingly, reversal of all the rejections of Claims 39-47, 49-52 and 55-58 is respectfully requested.

Please charge any additional fees, including any fees for additional extension of time, or credit overpayment to Deposit Account No. **50-4634** (referencing Attorney's Docket No. **123851-181890 (GNE-1618 P2C79)**).

Respectfully submitted,

Date: January 8, 2009

By:



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8. CLAIMS APPENDIX

Claims on Appeal

39. An isolated nucleic acid having at least 80% nucleic acid sequence identity to:
- (a) a nucleic acid sequence encoding the polypeptide of (SEQ ID NO: 290);
 - (b) a nucleic acid sequence encoding the polypeptide of (SEQ ID NO: 290), lacking its associated signal peptide;
 - (c) a nucleic acid sequence encoding the extracellular domain of the polypeptide of (SEQ ID NO: 290);
 - (d) the nucleic acid sequence of (SEQ ID NO: 289);
 - (e) the full-length coding sequence of the nucleic acid sequence of (SEQ ID NO: 289); or
 - (f) the full-length coding sequence of the cDNA deposited under ATCC accession number 209927.
40. The isolated nucleic acid of Claim 39 having at least 85% nucleic acid sequence identity to:
- (a) a nucleic acid sequence encoding the polypeptide of (SEQ ID NO: 290);
 - (b) a nucleic acid sequence encoding the polypeptide of (SEQ ID NO: 290), lacking its associated signal peptide;
 - (c) a nucleic acid sequence encoding the extracellular domain of the polypeptide of (SEQ ID NO: 290);
 - (d) the nucleic acid sequence of (SEQ ID NO: 289);
 - (e) the full-length coding sequence of the nucleic acid sequence of (SEQ ID NO: 289); or
 - (f) the full-length coding sequence of the cDNA deposited under ATCC accession number 209927.
41. The isolated nucleic acid of Claim 39 having at least 90% nucleic acid sequence identity to:
- (a) a nucleic acid sequence encoding the polypeptide of (SEQ ID NO: 290);

- (b) a nucleic acid sequence encoding the polypeptide of (SEQ ID NO: 290), lacking its associated signal peptide;
- (c) a nucleic acid sequence encoding the extracellular domain of the polypeptide of (SEQ ID NO: 290);
- (d) the nucleic acid sequence of (SEQ ID NO: 289);
- (e) the full-length coding sequence of the nucleic acid sequence of (SEQ ID NO: 289); or
- (f) the full-length coding sequence of the cDNA deposited under ATCC accession number 209927.

42. The isolated nucleic acid of Claim 39 having at least 95% nucleic acid sequence identity to:

- (a) a nucleic acid sequence encoding the polypeptide of (SEQ ID NO: 290);
- (b) a nucleic acid sequence encoding the polypeptide of (SEQ ID NO: 290), lacking its associated signal peptide;
- (c) a nucleic acid sequence encoding the extracellular domain of the polypeptide of (SEQ ID NO: 290);
- (d) the nucleic acid sequence of (SEQ ID NO: 289);
- (e) the full-length coding sequence of the nucleic acid sequence of (SEQ ID NO: 289); or
- (f) the full-length coding sequence of the cDNA deposited under ATCC accession number 209927.

43. The isolated nucleic acid of Claim 39 having at least 99% nucleic acid sequence identity to:

- (a) a nucleic acid sequence encoding the polypeptide of (SEQ ID NO: 290);
- (b) a nucleic acid sequence encoding the polypeptide of (SEQ ID NO: 290), lacking its associated signal peptide;
- (c) a nucleic acid sequence encoding the extracellular domain of the polypeptide of (SEQ ID NO: 290);

- (d) the nucleic acid sequence of (SEQ ID NO: 289);
- (e) the full-length coding sequence of the nucleic acid sequence of (SEQ ID NO: 289); or
- (f) the full-length coding sequence of the cDNA deposited under ATCC accession number 209927.

44. An isolated nucleic acid comprising:
- (a) a nucleic acid sequence encoding the polypeptide of (SEQ ID NO: 290);
 - (b) a nucleic acid sequence encoding the polypeptide of (SEQ ID NO: 290), lacking its associated signal peptide;
 - (c) a nucleic acid sequence encoding the extracellular domain of the polypeptide of (SEQ ID NO: 290);
 - (d) the nucleic acid sequence of (SEQ ID NO: 289);
 - (e) the full-length coding sequence of the nucleic acid sequence of (SEQ ID NO: 289); or
 - (f) the full-length coding sequence of the cDNA deposited under ATCC accession number 209927.

45. The isolated nucleic acid of Claim 44 comprising a nucleic acid sequence encoding the polypeptide of (SEQ ID NO: 290).

46. The isolated nucleic acid of Claim 44 comprising a nucleic acid sequence encoding the polypeptide of (SEQ ID NO: 290), lacking its associated signal peptide.

47. The isolated nucleic acid of Claim 44 comprising a nucleic acid sequence encoding the extracellular domain of the polypeptide of (SEQ ID NO: 290).

49. The isolated nucleic acid of Claim 44 comprising the nucleic acid sequence of (SEQ ID NO: 289).

50. The isolated nucleic acid of Claim 44 comprising the full-length coding sequence of the nucleic acid sequence of (SEQ ID NO: 289).

51. The isolated nucleic acid of Claim 44 comprising the full-length coding sequence of the cDNA deposited under ATCC accession number 209927.

52. An isolated nucleic acid molecule consisting of a fragment of the nucleic acid sequence of SEQ ID NO: 289, or a complement thereof, that specifically hybridizes under stringent conditions to:

- (a) a nucleic acid sequence encoding the polypeptide of (SEQ ID NO: 290);
- (b) a nucleic acid sequence encoding the polypeptide of (SEQ ID NO 290), lacking its associated signal peptide;
- (c) a nucleic acid sequence encoding the extracellular domain of the polypeptide of (SEQ ID NO: 290);
- (d) the nucleic acid sequence of (SEQ ID NO: 289);
- (e) the full-length coding sequence of the nucleic acid sequence of (SEQ ID NO: 289); or
- (f) the full-length coding sequence of the cDNA deposited under ATCC accession number 209927,

wherein said stringent conditions are hybridization in 50% formamide, 5 x SSC (0.75 M NaCl, 0.075 M sodium citrate), 50 mM sodium phosphate (pH 6.8), 0.1% sodium pyrophosphate, 5 x Denhardt's solution, sonicated salmon sperm DNA (50 µg/ml), 0.1% SDS, and 10% dextran sulfate at 42°C, with washes at 42°C in 0.2 x SSC (sodium chloride/sodium citrate) and 50% formamide at 55°C, followed by a high-stringency wash consisting of 0.1 x SSC containing EDTA at 55°C.

55. A vector comprising the nucleic acid of Claim 39.

56. The vector of Claim 55, wherein said nucleic acid is operably linked to control sequences recognized by a host cell transformed with the vector.

57. A host cell comprising the vector of Claim 55.
58. The host cell of Claim 57, wherein said cell is a CHO cell, an *E. coli* or a yeast cell.

9. EVIDENCE APPENDIX

1. Declaration of Sherman Fong, Ph.D. under 35 C.F.R §1.132, with attached Exhibits A-E:
 - A. Current Protocols in Immunology, Vol. 1, Richard Coico, Series Ed., John Wiley & Sons, Inc., 1991, Unit 3.12.
 - B. Steinman, R.M., "The dendritic cell advantage: New focus for immune-based therapies," *Drug News Perspect.* **13**:581-586 (2000).
 - C. Gubler, U. *et al.*, "Coexpression of two distinct genes is required to generate secreted bioactive cytotoxic lymphocyte maturation factor," *Proc. Natl. Acad. Sci. USA* **88**:4143-4147 (1991).
 - D. Peterson, A.C. *et al.*, "Immunization with melan-A peptide-pulsed peripheral blood mononuclear cells plus recombinant human interleukin-12 induces clinical activity and T-cell responses in advanced melanoma," *J. Clin. Oncol.* **21**:2342-2348 (2003).
 - E. Thurner, B. *et al.*, "Vaccination with Mage-3A1 peptide-pulsed mature, monocyte-derived dendritic cells expands specific cytotoxic T-cells and induces regression of some metastases in advanced stage IV melanoma," *J. Exp. Med.* **190**:1669-1678 (1999).
2. Kahan, Barry D., "Immunosuppressive therapy," *Curr. Opin. Immunol.* **4**:553-560 (1992).
3. Picotti, J.R. et al., "Interleukin-12 (IL-12)-driven alloimmune responses in vitro and in vivo," *Transplantation* **67**:1453-1460 (1999).
4. Campo, C.A. et al., "Zinc inhibits the mixed lymphocyte culture," *Biological Trace Element Research*, **79**:15-22 (2001)
5. Robins et al. US Patent 4,950,647
6. Strobel et al. US Patent 5,648,376
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12. Chapoval, A.I. et al., "Combination chemotherapy and IL-15 administration induces permanent tumor regression in a mouse lung tumor model: NK and T cell-mediated effects antagonized by B cells," J. Immunol. 161:6977-6984 (1998).
13. Fung-Leung, et al., "Tepoxalin, A Novel Immunomodulatory Compound, Synergizes with CSA in Suppression of Graft-Versus-Host Reaction and Allogenic Skin Graft Rejection", Transplantation, 60:362-368 (1995).
14. Gennari, R. et al., "Granulocyte macrophage colony stimulating factor improves survival in two models of gut-derived sepsis by improving gut barrier function and modulating bacterial clearance," Annals of Surgery 220:68-76 (1994).
15. Grabstein, K.H. et al., "Cloning of a T cell growth factor that interacts with the beta chain of the interleukin-2 receptor," Science 264:965-968 (1994).
16. Kasaian, M.T. et al., "IL-21 limits NK cell responses and promotes antigen specific T cell activation: a mediator of the transition from innate to adaptive immunity," Immunity 16:559-569 (2002).
17. Kirchner, G.I. et al., "Pharmacokinetics of recombinant human interleukin-2 in advanced renal cell carcinoma patients following subcutaneous application," Br. J. Clin. Pharmacol. 46:5-10 (1998).

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19. Naito, K. et al., "Macrophage factors which enhance the mixed leukocyte reaction initiated by dendritic cells," *J. Immunol.* 142:1834-1839 (1989).

20. Nastala, C.L. et al., "Recombinant IL-12 administration induces tumor regression in association with IFN- γ production," *J. Immunol.* 153:1697-1706 (1994).

21. Pahwa, R. et al. "Recombinant interleukin 2 therapy in severe combined immunodeficiency disease," *Proc. Natl. Acad. J Sci. USA* 86:5069-5073 (1989).

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Item 1 was submitted with Preliminary Amendment filed August 30, 2004, and was noted as considered by the Examiner on November 17, 2004.

Items 2-4 were made of record by the Examiner in the Office Action mailed May 30, 2006.

Items 5-22 were submitted with the Response filed November 3, 2006, and was noted as considered by the Examiner on February 1, 2007.

10. RELATED PROCEEDINGS APPENDIX

None - no decision rendered by a Court or the Board in any related proceedings identified above.

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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant: Docket No.:
Serial No.: Group Art Unit:
Filing Date: Examiner:
For:

DECLARATION OF SHERMAN FONG, Ph.D. UNDER 37 C.F.R. § 1.132

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

I, Sherman Fong, Ph.D. declare and say as follows: -

1. I was awarded a Ph.D. in Microbiology by the University of California at Davis, CA in 1975.
2. After postdoctoral training and holding various research positions at Scripps Clinic and Research Foundation, La Jolla, CA, I joined Genentech, Inc., South San Francisco, CA in 1987. I am currently a Senior Scientist at the Department of Immunology/Discovery Research of Genentech, Inc.
3. My scientific Curriculum Vitae is attached to and forms part of this Declaration.
4. I am familiar with the Mixed Lymphocyte Reaction (MLR) assay, which has been used by me and others under my supervision, to test the immune stimulatory or immune inhibitory activity of novel polypeptides discovered in Genentech's Secreted Protein Discovery Initiative project.
5. The MLR assay is a well known and widely used proliferative assay of T-cell function, the basic protocols of which are described, for example, in Current Protocols in Immunology Vol. 1, Richard Coico, Series Ed., John Wiley & Sons, Inc., 1991, Unit 3.12. (Exhibit A). This publication is incorporated by reference in the description of the MLR protocol in the present application.

6. The T-lymphocytes or "T-cells" of our immune system can be induced to proliferate by a variety of agents. The MLR assay is designed to study a particularly important induction mechanism whereby responsive T-cells are cultured together (or "mixed"), with other lymphocytes that are "allogeneic", e.g. lymphocytes that are taken from different individuals of the same species. In the MLR protocol of the present application, a suspension of PBMCs that includes responder T-cells, is cultured with allogeneic PBMCs that predominantly contain dendritic cells. According to the protocol, the allogeneic "stimulator" PBMCs are irradiated at a dose of 3000 Rad. This irradiation is done in order to create a sample of cells that has mainly dendritic cells. It is known that the dendritic cell population among the PBMCs are differentially affected by irradiation. At low doses (500-1000 Rad), the proliferation of most cells, including the B cells in the PBMCs, is preserved, however, at doses above 2000 Rad, this function of B cells is abolished. Dendritic cells on the other hand, maintain their antigen presentation function even at a 3000 Rad dose of radiation. (See, e.g. Current Protocols in Immunology, supra, at 3.12.9). Accordingly, under the conditions of the MLR assay used to test the PRO polypeptides of the present invention, the stimulator PBMCs remaining after irradiation are essentially dendritic cells.
7. Dendritic cells are the most potent antigen-presenting cells, which are able to "prime" naive T cells *in vivo*. They carry on their surface high levels of major histocompatibility complex (MHC) products, the primary antigens for stimulating T-cell proliferation. Dendritic cells provide the T-cells with potent and needed accessory or costimulatory substances, in addition to giving them the T-cell maturing antigenic signal to begin proliferation and carry out their function. Once activated by dendritic cells, the T-cells are capable of interacting with other antigen presenting B cells and macrophages to produce additional immune responses from these cells. For further details about the properties and role of dendritic cells in immune-based therapies see, e.g. Steinman, Drug News Perspect. 13(10):581-586 (Exhibit B).
8. The MLR assay of the present application is designed to measure the ability of a test substance to "drive" the dendritic cells to induce the proliferation of T-cells that are activated, or co-stimulated in the MLR, and thus identifies immune stimulants that can boost the immune system to respond to a particular antigen that may not have been immunologically active previously.

9. Such immune stimulants find important clinical applications. For example, IL-12 is a known immune stimulant, which has been shown to stimulate T-cell proliferation in the MLR assay. IL-12 was first identified in just such an MLR [Gubler et al. PNAS 88, 4143 (1991) (Exhibit C)]. In a recent cancer vaccine trial, researchers from the University of Chicago and Genetics Institute (Cambridge, MA) have demonstrated the efficacy of the approach, relying on the immune stimulatory activity of IL-12, for the treatment of melanoma. [Peterson et al. Journal of Clinical Oncology 21 (12). 2342-48 (2003) (Exhibit D)] They extracted circulating white blood cells carrying one or more markers of melanoma cells, isolated the antigen, and returned them to the patients. Normally patients would not have an immune response to his or her own human antigens. The patients were then treated with different doses of IL-12, an immune stimulant capable of inducing the proliferation of T cells that have been co-stimulated by dendritic cells. Due to the immune stimulatory effect of IL-12, the treatment provided superior results in comparison to earlier work, where patients' own dendritic cells were prepared from peripheral blood mononuclear cells (PBMCs), treated with antigens, then cultured *in vitro* and returned to the patient to stimulate anti-cancer response. [Thurner et al. J. Exp. Med. 190 (11), 1669-78 (1999) (Exhibit E)].
10. It is my considered scientific opinion that a PRO polypeptide shown to stimulate T-cell proliferation in the MLR assay of the present invention with an activity at least 180% of the control, as specified in the present application, is expected to have the type of activity as that exhibited by IL-12, and would therefore find practical utility as an immune stimulant. Some PRO polypeptides do the reverse, and give inhibition of T-cell proliferation in the MLR assay. It is my considered scientific opinion that a PRO polypeptide shown to inhibit T-cell proliferation in the MLR assay where the activity is observed as 80% or less of the control, as specified in the present application, would be expected to find practical utility when an inhibition of the immune response is desired, such as in autoimmune diseases.

Dated: 6/14/04

By: Sherman Fong

Sherman Fong, Ph.D.

Sherman Fong, Ph.D.

Senior Scientist

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Education:

1978 - 1980 Postdoctoral Fellow in Immunology, Research Institute of Scripps Clinic,
Scripps Clinic and Research Foundation, La Jolla, California

1975 - 1978 Postdoctoral Fellow in Immunology, University of California at
San Francisco, San Francisco, California

1970 - 1975 Ph.D. in Microbiology, University of California at
Davis, California

1966 - 1970 B.A. in Biology/Microbiology, San Francisco State
University, San Francisco, California

Professional Positions:

Currently: Senior Scientist, Department of Immunology/Discovery Research, Genentech, Inc., South San Francisco, California

8/00-8/01 Acting Director, Department of Immunology, Genentech, Inc. South San Francisco, California

10/89 Senior Scientist in the Department of Immunology/Discovery Research, Genentech, Inc.
South San Francisco, California

3/89 - 10/89 Senior Scientist and Immunobiology Group Leader, Department of Pharmacological Sciences, Immunobiology Section/Medical Research and Development, Genentech, Inc., S. San Francisco, California

9/87 - 3/89 Scientist, Department of Pharmacological Sciences, Immunopharmacology Section/Medical Research and Development, Genentech, Inc., S. San Francisco, California

1/82 - 9/87 Assistant Member (eq. Assistant Professor level), Department of Basic and Clinical Research, Division of Clinical Immunology, Scripps Clinic and Research Foundation, La Jolla, California

6/80 - 12/81 Scientific Associate in the Department of Clinical Research, Division of Clinical Immunology, Scripps Clinic and Research Foundation, La Jolla, California

7/78 - 6/80 Postdoctoral training in the laboratory of Dr. J. H. Vaughan, Chairman, Department of Clinical Research, Division of Clinical Immunology, Scripps Clinic and Research Foundation, La Jolla, California

2/75 - 6/78 Postdoctoral training in the laboratory of Dr. J. W. Goodman, Department of Microbiology and Immunology, School of Medicine, University of California, San Francisco, California

7/71 - 12/74 Research Assistant and Graduate Student, Department of Medical Microbiology, School of Medicine, University of California, Davis, California, under Dr. E. Benjamini

Awards:

Recipient: National Institutes of Health Postdoctoral Fellowship Award (1975).

Recipient: Special Research Award, (New Investigator Award), National Institute of Health (1980).

Recipient: P.I., Research Grant Award, National Institute of Health (1984).

Recipient: Research Career Development Award (R01), National Institutes of Health (1985).

Recipient: P.I., Multi-Purpose Arthritis Center Research Grant, NIH (1985)

Recipient: P.I., Research Grant Award, (R01 Renewal), National Institute of Health (1987).

Scientific Associations:

Sigma Xi, University of California, Davis, California Chapter

Member, The American Association of Immunologists

Committee Service and Professional Activities:

Member of the Immunological Sciences Study Section, National Institutes of Health Research Grant Review Committee, (1988-1992).

Advisory Committee, Scientific Review Committee for Veteran's Administration High Priority Program on Aging, 1983.

Ad Hoc member of Immunological Sciences Study Section, National Institutes of Health, 1988.

Ad Hoc Reviewer: Journal of Clinical Investigations, Journal of Immunology, Arthritis and Rheumatism, International Immunology, Molecular Cell Biology, and Gastroenterology

Biotechnology Experience

Established at Genentech in 1987-1989 within the Immunobiology Laboratory, in the Department of Pharmacological Sciences, group to study the immunogenicity of recombinant hGH (Protropin®) in hGH transgenic mice.

Served as Immunologist on the Biochemical Subteam for Protropin® Project team.

Served as Immunologist on the Met-less hGH and Dnase project teams, two FDA approved biological drugs: second generation hGH Nutropin® and Pulmozyme® (DNase).

Served immunologist in 1989-1990 on the CD4-IgG project team carrying out in vitro immunopharmacological studies of the effects of CD4-IgG on the in vitro human immune responses to mitogens and antigens and on neutrophil responses in support of the filing of IND to FDA in 1990 for use of CD4-IgG in the prevention of HIV infection. Product was dropped.

In 1989-1991, initiated and carried research and development work on antibodies to CD11b and CD18 chains of the leukocyte β2 integrins. Provided preclinical scientific data to Anti-CD18 project team

supporting the advancement of humanized anti-CD18 antibody as anti-inflammatory in the acute setting. IND filed in 1996 and currently under clinical evaluation.

1993-1997, **Research Project Team leader** for small molecule $\alpha 4\beta 1$ integrin antagonist project. Leader for collaborative multidisciplinary team (N=11) composed of immunologists, molecular/cell biologists, protein engineers, pathologists, medicinal chemists, pharmacologists, pharmaceutical chemists, and clinical scientists targeting immune-mediated chronic inflammatory diseases. Responsible for research project plans and execution of strategy to identify lead molecules, assessment of biological activities, preclinical evaluation in experimental animals, and identification of potential clinical targets. Responsible for identification, hiring, and working with outside scientific consultants for project. Helped establish and responsible for maintaining current research collaboration with Roche-Nutley. Project transferred to Roche-Nutley.

1998-present, worked with Business Development to identify and create joint development opportunity with LeukoSite (currently Millennium) for monoclonal antibody against $\alpha 4\beta 7$ intergrin (LDP-02) for therapeutic treatment for inflammatory bowel disease (UC and Crohn's disease). Currently, working as scientific advisor to the core team for phase II clinical trials for LDP-02.

Currently, **Research Project Team Biology Leader** (1996-present) for small molecule antagonists for $\alpha 4\beta 7$ /MAdCAM-1 targeting the treatment of human inflammatory bowel diseases and diseases of the gastrointestinal tract. Responsible for leading collaborative team (N=12) from Departments of Immunology, Pathology, Analytical Technology, Antibody Technology, and Bio-Organic Chemistry to identify and evaluate lead drug candidates for the treatment of gastrointestinal inflammatory diseases.

Served for nearly fifteen years as **Ad Hoc reveiwer** on Genentech Internal Research Review Committee, Product Development Review Committee, and Pharmacological Sciences Review Committee.

Worked as **Scientific advisor** with staff of the **Business Development Office** on numerous occasions at Genentech, Inc. to evaluate the science of potential in-licensing of novel technologies and products.

2000-2001 Served as Research Discovery representative on Genentech Therapeutic Area Teams (Immunology/Endocrine, Pulmonary/Respiratory Disease Task Force)

Invited Symposium Lectures:

Session Chairperson and speaker, American Aging Association 12th Annual National Meeting, San Francisco, California, 1982.

Invited Lecturer, International Symposium, Mediators of Immune Regulation and Immunotherapy, University of Western Ontario, London, Ontario, Canada, 1985.

Invited Lecturer, workshop on Human IgG Subclasses, Rheumatoid Factors, and Complement. American Association of Clinical Chemistry, San Francisco, California, 1987.

Plenary Lecturer, First International Waaler Conference on Rheumatoid Factors, Bergen, Norway, 1987.

Invited Lecturer, Course in Immunorheumatology at the Universite aux Marseilles, Marseilles, France, 1988.

Plenary Lecturer, 5th Mediterranean Congress of Rheumatology, Istanbul, Turkey, 1988.

Invited Lecturer, Second Annual meeting of the Society of Chinese Bioscientist of America, University of California, Berkeley, California, 1988.

Lecturer at the inaugural meeting of the Immunology by the Bay sponsored by The Bay Area Bioscience Center. The β 2 Integrins in Acute Inflammation, July 14, 1992.

Lecturer, "Research and Development -- An Anatomy of a Biotechnology Company", University of California, Berkeley, Extension Course, given twice a year--March 9, 1995 to June 24, 1997.

Lecturer, "The Drug Development Process - Biologic Research - Genomics", University of California, Berkeley Extension, April 21, 1999, October, 1999, April 2000, October, 2000.

Lecturer, "The Drug Development Process - Future Trends/Impact of Pharmacogenomics", University of California Berkeley Extension, April 2001, October 2001, April 2002.

Invited Speaker, "Targeting of Lymphocyte Integrin $\alpha 4\beta 7$ Attenuates Inflammatory Bowel Diseases", in Symposium on "Nutrient effects on Gene Expression" at the Institute of Food Technology Symposium, June, 2002.

Patents:

Dennis A. Carson, Sherman Fong, Pojen P. Chen.

U.S. Patent Number 5,068,177: Anti-idiotype Antibodies induced by Synthetic Polypeptides, Nov. 26, 1991

Sherman Fong, Caroline A. Hebert, Kyung Jin Kim and Steven R. Leong.

U.S. Patent Number 5,677,426: Anti-IL-8 Antibody Fragments, Oct. 14, 1997

Claire M. Doerschuk, Sherman Fong, Caroline A. Hebert, Kyung Jin Kim, Steven R. Leong. U.S. Patent Number 5,686,070: Methods for Treating Bacterial Pneumonia, Nov. 11, 1997

Claire M. Doerschuk, Sherman Fong, Caroline A. Hebert, Kyung Jin Kim, Steven R. Leong. U.S. Patent 5,702,946: Anti-IL-8 Monoclonal Antibodies for the Treatment of Inflammatory Disorders, Dec. 30, 1997

Sherman Fong, Caroline A. Hebert, Kyung Jin Kim, Steven R. Leong.

U.S. Patent Number 5,707,622: Methods for Treating Ulcerative Colitis, Jan. 13, 1998

Sherman Fong, Napoleone Ferrara, Audrey Goddard, Paul Godowski, Austin Gurney, Kenneth Hillan, and Mickey Williams. U.S. Patent Number 6,074,873: Nucleic acids encoding NL-3, June 13, 2000

Sherman Fong, Napoleone Ferrara, Audrey Goddard, Paul Godowski, Austin Gurney, Kenneth Hillan, and Mickey Williams. U.S. Patent Number 6,348,351 B1: The Receptor Tyrosine Kinase Ligand Homologues. February 19, 2002

Patent Applications:

Sherman Fong, Kenneth Hillan, Toni Klassen

U.S. Patent Application: "Diagnosis and Treatment of Hepatic Disorders"

Sherman Fong, Audrey Goddard, Austin Gurney, Daniel Tumas, William Wood

U.S. Patent Application: Compositions and Methods for the Treatment of Immune Related Diseases.

Sherman Fong, Mary Gerritsen, Audrey Goddard, Austin Gurney, Kenneth Hillan, Mickey Williams, William Wood. U.S. Patent Application: Promotion or Inhibition of Cardiovasculogenesis and Angiogenesis

Avi Ashkenazi, Sherman Fong, Audrey Goddard, Austin Gurney, Mary Napier, Daniel Tumas, William Wood. US Patent Application: Compounds, Compositions and Methods for the Treatment of Diseases Characterized by A33-Related Antigens

Chen, Filvaroff, Fong, Goddard, Godowski, Grimaldi, Gurney, Hillan, Tumas, Vandlen, Van Lookeren, Watanabe, Williams, Wood, Yansura

US Patent Application: IL-17 Homologous Polypeptides and Therapeutic Uses Thereof

Ashkenazi, Botstein, Desnoyers, Eaton, Ferrara, Filvaroff, Fong, Gao, Gerber, Gerritsen, Goddard, Godowski, Grimaldi, Gurney, Hillan, Kljavin, Mather, Pan, Paoni, Roy, Stewart, Tumas, Williams, Wood
US Patent Application: Secreted And Transmembrane Polypeptides And Nucleic Acids Encoding The Same

Publications:

1. Scibienski R, Fong S, Benjamini E: Cross tolerance between serologically non-cross reacting forms of egg white lysozyme. *J Exp Med* 136:1308-1312, 1972.
2. Scibienski R, Harris M, Fong S, Benjamini E: Active and inactive states of immunological unresponsiveness. *J Immunol* 113:45-50, 1974.
3. Fong S: Studies on the relationship between the immune response and tumor growth. Ph D Thesis, 1975.
4. Benjamini E, Theilen G, Torten M, Fong S, Crow S, Henness AM: Tumor vaccines for immunotherapy of canine lymphosarcoma. *Ann NY Acad Sci* 277:305, 1976.
5. Benjamini E, Fong S, Erickson C, Leung CY, Rennick D, Scibienski RJ: Immunity to lymphoid tumors induced in syngeneic mice by immunization with mitomycin C treated cells. *J Immunol* 118:685-693, 1977.
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14. Tsoukas CD, Fox RI, Slovin SF, Carson DA, Pellegrino M, Fong S, Pasquali J-L, Ferrone S, Kung P, Vaughan JH: T lymphocyte-mediated cytotoxicity against autologous EBV-genome-bearing B cells. *J Immunol* 126:1742-1746, 1981.
15. Fong S, Tsoukas CD, Pasquali J-L, Fox RI, Rose JE, Raiklen D, Carson DA, Vaughan JH: Fractionation of human lymphocyte subpopulations on immunoglobulin coated petri dishes. *J Immunol Methods* 44:171-182, 1981.
16. Pasquali J-L, Tsoukas CD, Fong S, Carson DA, Vaughan JH: Effect of Levamisole on pokeweed mitogen stimulation of immunoglobulin production in vitro. *Immunopharmacology* 3:289-298, 1981.

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23. Tsoukas CD, Fox RI, Carson DA, Fong S, Vaughan JH: Molecular interactions in human T-cell-mediated cytotoxicity to Epstein-Barr virus. I. Blocking of effector cell function by monoclonal antibody OKT3. *Cell Immunol* 69:113-121, 1982.
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25. Fox RI, Carstens SA, Fong S, Robinson CA, Howell F, Vaughan JH: Use of monoclonal antibodies to analyze peripheral blood and salivary gland lymphocyte subsets in Sjogren's Syndrome. *Arthritis Rheum* 25:419, 1982.
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40. Fox RI, Adamson III TC, Fong S, Robinson CA, Morgan EL, Robb JA, Howell FV: Lymphocyte phenotype and function in pseudolymphoma associated with Sjogren's syndrome. *J Clin Invest* 72:52-62, 1983.
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42. Chen PP, Houghten RA, Fong S, Rhodes GH, Gilbertson TA, Vaughan JH, Lerner RA, Carson DA: Anti-hypervariable region antibody induced by a defined peptide. A new approach for studying the structural correlates of idiotypes. *Proc Natl Acad Sci USA* 81:1784-1788, 1984.
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46. Fong S: Immunochemistry. In: Immunology as applied to Otolaryngology. Ryan AF, Poliquis JF, Harris A (eds.): pp. 23-53. College Hill Press, San Diego, 1985.
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Proliferative Assays for T Cell Function

UNIT 3.12

A number of agents can specifically or nonspecifically induce T cell activation, resulting in cytokine production, cytokine receptor expression, and ultimately proliferation of the activated T cells. Although proliferation is not a specific effector function of T lymphocytes—in contrast to helper function for B lymphocytes (*UNIT 3.10*) or cytotoxicity (*UNIT 3.11*)—proliferation assays are reliable, simple, and easy to perform and have been widely used to assess the overall immunocompetence of an animal. In addition, the assays described in this unit form the basis for identifying the appropriate cellular population that might be used to obtain T cell clones (*UNIT 3.13*) or T cell hybridomas (*UNIT 3.14*).

The assays have been divided into two groups on the basis of whether they are used to stimulate primed or unprimed T lymphocytes. The first basic protocol describes the use of agents that are capable of activating unprimed T lymphocytes in culture either by pharmacologic means (calcium ionophore and phorbol ester stimulation), by direct cross-linking of the T cell receptor (TCR) on a large percentage of responder cells (anti-CD3, anti-TCR- $\gamma\delta$, or anti-TCR- $\alpha\beta$ monoclonal antibodies), by cross-linking the receptors on certain subpopulations of T cells with monoclonal antibodies specific for the V regions of β chains of the TCR (anti-V β) or with enterotoxins specific for certain V β -chain regions, or by indirectly cross-linking the TCR (lectins or monoclonal antibodies to non-TCR antigens). The first alternate protocol describes the use of plate-bound antibodies specific for the TCR to stimulate proliferation. The second alternate protocol describes the activation of unprimed T cells to cell-associated antigens in the mixed leukocyte reaction (MLR). The first support protocol describes the preparation and use of T cell-depleted accessory or stimulator cells and the second support protocol describes methods for blocking accessory cell proliferation. Finally, the second basic protocol describes the induction of a T cell proliferative response to soluble protein antigens or to cell-associated antigens against which the animal has been primed *in vivo*.

The assays in this unit employ murine T lymphocytes. Induction of proliferative responses of murine B lymphocytes is described in *UNIT 3.10*. Related assays for use with human peripheral blood lymphocytes are described in *UNIT 7.9*.

NOTE: All solutions and equipment coming into contact with cells must be sterile, and proper sterile technique should be used accordingly.

ACTIVATION OF UNPRIMED T CELLS

Unprimed T cells can be induced to proliferate by a variety of agents, including pharmacological agents, anti-CD3/TCR or anti-Thy-1 monoclonal antibodies, enterotoxins and lectins. The commentary briefly describes the specificities of these agents, while Table 3.12.1 lists sources and concentrations for use in this protocol. Although this procedure is intended to measure proliferation of T cells specifically, in many cases induction of T cell proliferation is dependent on the presence of non-T cells that function as accessory cells. The latter provide additional costimulatory signals for T cell proliferation as well as cross-link (via their Fc receptors) monoclonal antibodies bound to cell-surface antigens. The requirement for non-T accessory cells varies with the nature of the stimulatory ligand and can range from absolute dependence to accessory cell-independent T cell activation (see Table 3.12.1). The activation is calculated after determining the difference in incorporation of [³H]thymidine between stimulated and control cells.

BASIC PROTOCOL

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Table 3.12.1 Agents Used to Activate Unprimed T Cells in Proliferative Assays

Agent ^a	Source/ cat. no. ^b	Concentration	Accessory cells ^c	Mode of action, etc.
PMA	SIG P8139	1-10 ng/ml	No	Use with ionomycin or A23187; pharmacologic
Ionomycin	CAL 407950	200-500 ng/ml	No	Use with PMA; pharmacologic
A23187	CAL 100105	100-500 ng/ml	No	Use with PMA; pharmacologic
PHA	WD HA16	1-5 µg/ml	Yes	Indirect TCR cross-linking
Con A	PH 17-0450-01	1-10 µg/ml	Yes	Indirect TCR cross-linking
Anti-Thy-1	PG mAb-G7	1-50 µg/ml	Yes ^c	Indirect TCR cross-linking
Anti-CD3	PG HM-CD3	0.1-5 µg/ml	Yes ^c	Use plate-bound or soluble; direct TCR cross-linking
Anti-TCR-αβ	PG HM-AB-TCR	0.1-10 µg/ml	Yes ^c	Use plate-bound or soluble; direct TCR cross-linking
Anti-TCR-γδ	PG HM-GD-TCR-1; HM-GD-TCR-3	0.1-100 µg/ml	No	Use plate-bound; direct TCR cross-linking
Anti-Vβ-8.1, 8.2 ^c	PG MM-Vβ-TCR-1	0.1-100 µg/ml	No	Use plate-bound; direct TCR cross-linking
Anti-Vβ-6 ^c	PG RM-Vβ-TCR-2	0.1-100 µg/ml	No	Use plate-bound; direct TCR cross-linking
Anti-Vβ-11	PG RM-Vβ-TCR-3	0.1-100 µg/ml	No	Use plate-bound; direct TCR cross-linking
Staph tox A	TT AT101	1-10 µg/ml	Yes ^c	Vβ-1,3,10,11,17-receptor specificity
Staph tox B	TT BT202; SIG S4881	1-100 µg/ml	Yes ^c	Vβ-3,7,8,17-receptor specificity
Staph tox E	TT ET404	1-10 µg/ml	Yes ^c	Vβ-11,15,17-receptor specificity

^aAbbreviations: PMA, phorbol 12-myristate 13-acetate; PHA, phytohemagglutinin; Con A, concanavalin A; Staph tox A, B, & E, *Staphylococcus* enterotoxins A, B, & E.

^bSupplier addresses and phone numbers are provided in APPENDIX 5. Abbreviations: CAL, Calbiochem; PG, Pharmingen; PH, Pharmacia LKB; SIG, Sigma; TT, Toxin Technology; WD, Wellcome Diagnostics.

^cWhen using anti-CD3 and anti-TCR antibodies in soluble form (rather than plate-bound), accessory cells are required. When using Staph enterotoxins, accessory cells must express appropriate MHC class II molecules. Accessory cell dependence is not absolute with anti-Thy-1 antibodies.

Materials

Complete RPMI-5 and RPMI-10 media (*APPENDIX 2*)

Responder cells: lymphocytes from nonimmunized mouse thymus, spleen, or lymph nodes (*UNIT 3.1*)

Activating agent(s) (Table 3.12.1)

Phosphate-buffered saline (PBS; *APPENDIX 2*)

Accessory cells: unfractionated mouse spleen cell suspension, irradiated or treated with mitomycin C (second support protocol) or T cell-depleted (first support protocol)

[³H]thymidine (*APPENDIX 3*)

15- and 4-ml disposable, polystyrene conical tubes with screw caps

Low-speed centrifuge with Sorvall H-1000B rotor (or equivalent)

1-, 5-, and 10-ml disposable polystyrene pipets

96-well flat- or round-bottom microtiter plates with lids (Costar #3596 or #3799)

25- to 100- μ l single- and multichannel pipettors with disposable tips

Additional reagents and equipment for removing organs (*UNIT 1.9*), preparing single-cell suspensions (*UNIT 3.1*), and counting, labeling, and harvesting cells (*APPENDIX 3*)

1. Prepare responder leukocyte suspensions from thymus, spleen, or lymph node in complete RPMI-5 as described in *UNIT 3.1*.

The size of the intended experiment dictates the number of organs to be collected. See annotation to step 3 for an indication of cell number required, and UNIT 3.1 for number of cells per organ. Spleen, thymus, and lymph node can be used as responder cells, while only spleen is a source of accessory cells. Purified T cells or subpopulations of T cells (i.e., CD4⁺ or CD8⁺) cells may also be used. See UNITS 3.1-3.6 for enrichment/depletion methods.

2. Centrifuge single-cell suspensions in 15-ml conical tubes for 10 min in Sorvall H-1000B rotor at ~1000 rpm (200 \times g), room temperature, and discard supernatant.
3. Resuspend cell pellet in complete RPMI-5. Count responder cells and adjust to $\sim 10^6$ cells/ml with complete RPMI-10.

While this concentration (1 \times 10⁶ cells/ml or 2 \times 10⁵ cells/well) will give satisfactory responses with most cell populations, it is useful to compare 2, 4, and 8 \times 10⁵ cells per well in initial pilot experiments. If unfractionated spleen or lymph node cells are used as the responder population, sufficient accessory cells are present and there is no need to supplement the cultures with additional cells. However, if highly purified T cells or T cell subpopulations are used as responders, it will be necessary to add non-T accessory cells depending on the nature of the activating agent (see Table 3.12.1). This is most easily accomplished by adding increasing numbers (0.1, 0.5, and 1.0 \times 10⁵) of syngeneic spleen (accessory) cells in 0.1 ml to 2 \times 10⁵ T cells in 0.1 ml (see first support protocol). Also, a meaningful comparison of the responsiveness of different cell populations requires titrations of both the activating agents as well as the responding cell populations, and a kinetic experiment.

4. Prepare working solutions of activating agents in 4-ml conical tubes at room temperature as follows. For MAb, toxin, or lectin, make a series of four dilutions from 1 mg/ml stock solutions—e.g., 100, 30, 10, and 3 μ g/ml in PBS. For the pharmacological agent, make single dilutions of 100 ng/ml solution of PMA and 1 μ g/ml A23187 (or 4 μ g/ml ionomycin) in PBS.

If MAb in supernatant or ascites form are being used, at least four dilutions should also be used. Working solutions should be used immediately, since the various proteins, especially MAb, may bind to the plastic.

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See Table 3.12.1 for V β specificities of staphylococcal enterotoxins. It is essential to verify that the mouse strain employed expresses the MHC class II surface molecules for which the enterotoxin has a specific binding affinity. See Marrack and Kappler (1989) for further discussion of various enterotoxins and their specificities.

5. Add 20 μ l of each dilution of activating reagent (MAb, enterotoxin or lectin) to each of three wells of a 96-well flat- or round-bottom microtiter plate. Include control wells with 20 μ l of PBS only. Add 20 μ l PMA or calcium ionophore at the single concentration indicated in step 4, as the dose-response curve for these agents is extremely narrow.

A series of four dilutions will form one row of each microtiter plate, allowing for efficient organization of the plates.

6. To the wells of the 96-well microtiter plate containing activating agent, add 2×10^5 cells in 0.2 ml.
7. Place microtiter plates in a humidified 37°C, 5% CO₂ incubator for 2 to 4 days.

Optimum culture periods for stimulating cells will vary depending on cell type and laboratory conditions and must be determined empirically (see critical parameters).

8. Add [³H]thymidine to each well. Return the plates to CO₂ incubator to pulse 18 to 24 hr. Harvest cells using a semiautomated sample harvester and measure cpm in β scintillation counter.
- 9a. Compute the data as the difference in cpm of stimulated (experimental) and control (no activating agent added) cultures. This is done by subtracting the arithmetic mean of cpm from triplicate control cultures from the arithmetic mean of cpm from corresponding stimulated cultures. The results are referred to as " Δ cpm."
- 9b. Alternatively, compute the data as the ratio of cpm of stimulated and control cultures. This is done by dividing the arithmetic mean of cpm from stimulated cultures by the arithmetic mean of cpm from control cultures. The results are referred to "SI" (stimulation index).

The second method (step 9b) has the disadvantage that small changes in background values will result in large changes in SI and should be interpreted with caution. In most publications, Δ cpm rather than SI values are preferred.

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ACTIVATION OF UNPRIMED T CELLS WITH PLATE-BOUND ANTIBODIES

Although it is possible to induce T cell activation with monoclonal antibodies to the CD3/TCR complex in solution during culture, such activation depends on cross-linking of the antibody by Fc receptor-bearing accessory cells. This protocol describes the use of monoclonal antibodies to the CD3/TCR complex by coupling them to the wells of the microtiter plates. The T cell proliferative response induced under these conditions does not require the presence of significant numbers of accessory cells, although the responses obtained may be suboptimal (Jenkins et al., 1990).

Use of this protocol is recommended for use with those antibodies to the CD3/TCR complex which bind poorly to the Fc receptor present on murine accessory cells and which do not induce T cell activation in soluble form. Although all monoclonal antibodies readily couple to plastic under these conditions, it is very difficult to induce a proliferative response with certain antibodies such as the G7, anti-Thy-1 monoclonal antibody. In such cases, the conditions described in the basic protocol should be followed.

Additional Materials

PBS (APPENDIX 2), room temperature and 4°C

1 mg/ml purified anti-CD3 or anti-TCR MAb in PBS (for nonspecific activation of T cells) or 1 mg/ml purified anti-V β or anti-TCR- $\gamma\delta$ MAb in PBS (for activation of T cells with specific receptors; see Table 3.12.1)

1. In 4-ml conical polystyrene tubes, prepare a series of four dilutions of MAb from sterile 1 mg/ml stock solutions—e.g., 100, 10, 1, and 0.1 μ g/ml—using room temperature PBS.

Sources and recommended concentrations of monoclonal antibodies can be found in Table 3.12.1; since MAb will bind to plastic, the working dilutions should be used immediately.

The ability of anti-TCR antibodies to cross-link receptor molecules varies depending on the purity of the MAb preparation and the affinity of the MAb for the TCR/CD3 complex. Optimum dilutions will have to be determined in dose-response experiments. Alternatively, preparations of ascites fluid from the MAb can be tested at different dilutions (e.g., 1:100, 1:200, 1:400, and 1:800), but use of purified antibody will allow for better standardization of the assay.

Because the efficacy of MAb-induced activation depends on the amount of antibody bound to the bottom of the wells, it is crucial to make the dilutions in a buffer without any additional source of proteins such as FCS or albumin; these would compete with the binding of the antibody, and therefore reduce the responsiveness. For this reason, it is also not recommended to perform the assay with culture supernatants of the appropriate hybridomas.

2. Add 30 μ l of each concentration of MAb solution to each of three wells of a 96-well round-bottom microtiter plate. Include control wells of 30 μ l PBS only.

A series of four dilutions will form one row of each plate, allowing for efficient organization of the plates. Consistently better responses are seen with round-bottom (compared with flat-bottom) plates in antibody-mediated experiments.

Most often, optimal responses are seen with 10 μ g/ml antibody. There is no point in adding more than the indicated amount of antibody, since the maximum amount that can bind to surface of the wells is ~2 to 3 μ g (A.M.K., unpub. observ.).

3. Cover the plate and gently tap its side to ensure complete covering of the bottom of the wells. Incubate plates 90 min at 37°C. During incubation, proceed to step 4.

During this incubation, the antibodies bind to the plastic in the wells for subsequent cross-linking of the T cell receptors on responding T cells. Plates can also be prepared the night before an experiment and kept in the refrigerator overnight, after the 37°C incubation.

4. Prepare responder cell suspensions as in steps 1 to 3 of the basic protocol.

Highly purified T cell populations can be used in these studies as the proliferative response induced is accessory cell-independent. However, the presence of non-T accessory cells does not interfere with the proliferative response.

5. Wash the wells of the incubated plates by adding 200 μ l cold PBS and inverting the plates with a flick of the hand on a stack of paper towels placed in a tissue culture hood. Repeat washing procedure two more times to remove excess antibody.

6. To the wells of the washed plates, add $\sim 2 \times 10^5$ cells in 0.2 ml.

If cells are not ready at this stage, plates may be kept in the refrigerator overnight after 100 μ l PBS has been added. Presumably, longer storage periods should be acceptable, but our experience is limited to ≤ 4 day periods. The PBS should be removed before the cells are added.

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Most cell populations will give peak responsiveness at this cell dosage, but pilot experiments should be performed to establish optimal conditions.

7. Proceed as in steps 7 to 9 of the basic protocol, but incubate cultures for 2 to 3 days before adding [³H]thymidine.

Kinetic assays should be performed to determine the optimum culture period.

T CELL PROLIFERATION IN MIXED LYMPHOCYTE CULTURES

In the mixed lymphocyte culture (MLC) or reaction (MLR), suspensions of responder T cells are cultured with allogeneic stimulator lymphocytes. The activating stimulus is the foreign histocompatibility antigen (usually MHC class I or class II molecules) expressed on the allogeneic stimulator cells. Responder cells need not be primed because a sufficiently high number of T cells in the MLC will respond to the stimulator population. If the stimulator cell population contains T cells, their uptake of [³H]thymidine must be prevented by irradiation or treatment with mitomycin C; alternatively the stimulator cell suspension can be depleted of T cells (see support protocols).

Additional Materials

Responder cells: lymphocytes from nonimmunized mouse thymus, spleen, or lymph nodes (*UNITS 1.9 & 3.1*) or purified T cells or T cell subpopulations (*UNITS 3.1-3.6*)

Stimulator cells: allogeneic mouse spleen cells that differ from the responder cells at H-2 or Mls loci, irradiated or treated with mitomycin C (second support protocol) or T cell-depleted (first support protocol)

1. Prepare responder cell populations as in steps 1 to 3 of the basic protocol. Although unfractionated cell populations can be used as responders in certain situations, it may be preferable to use purified T cells or T cell subsets.

To estimate the MLR of a cell population, it is necessary to perform a dose-response assay with different numbers of responder cells. Typically, three replicate wells are set up containing each of the following: 0.5, 1, 2, and 4 × 10⁵ cells (optimal responses are usually obtained with the latter two densities). The setup for these four cell densities will occupy one row (12 wells) of a microtiter plate.

For thymocytes, it may be necessary to use 8 × 10⁵ cells per well because the frequency of responding T cells is lower; the lowest number of responder cells could then be 1 × 10⁵ and the doses in between would be 2 and 4 × 10⁵. Using this range of higher numbers of responder cells may also be preferred when experimental manipulations are expected to reduce the frequency of responding T cells.

2. To a 96-well microtiter plate, add 5 × 10⁴ to 4 × 10⁵ responder cells in 0.1 ml to each well. For each experimental group, set up three replicate wells.

*Stimulation of leukocytes for proliferation in 96-well microtiter plates can be run in parallel with cytotoxic T lymphocyte (CTL) generation (*UNIT 3.11*), which is performed in 24-well microtiter plates. For example, cells can be diluted to 4 × 10⁶ cells/ml and added to 24-well plates in 1.0 ml/well for CTL generation and to 96-well plates in 0.1 ml/well for proliferation.*

3. Prepare a single-cell suspension of irradiated or mitomycin C-treated stimulator cells. Alternatively, prepare a suspension of T-cell depleted stimulator cells. Add 0.1 ml to each well of the plates containing responder cells.

The optimum number of stimulator cells must be determined for each MLC and for different responder cells. For a range of responder cells from 0.5-4 × 10⁵, test stimulator cells at densities of 2, 4, and 8 × 10⁶/ml (i.e., 2, 4, and 8 × 10⁵/well). It should be noted that the stimulator cell suspension provides both the specific antigen to be recognized by the responder T cells as well as nonspecific accessory cells. If

highly purified T cells are used as the responder population, it is therefore not necessary to supplement the cultures with non-T accessory cells syngeneic to the responder T cells.

Separate wells with control cultures should be set up that include—for each dose of responder and stimulator cells—replicate wells of responder cells with irradiated or mitomycin C-treated syngeneic stimulator cells. Values obtained from these controls reflect "background" proliferation values (see step 9 of basic protocol). Other negative controls often included are wells with stimulator cells alone and wells with responder cells alone. These are not used for the calculation of the data, but are useful to compare with the background proliferation values; the latter should not be much higher (<2-fold) than those obtained with stimulator or responder cells alone. Higher background values indicate potential autoreactivity.

4. Follow steps 7 to 9 of the basic protocol, but incubate the cultures for 3 to 6 days.

Optimum culture periods for stimulating cells will vary depending on cell type and laboratory conditions, and must be determined empirically (see critical parameters).

DEPLETION OF T CELLS FROM ANTIGEN-PRESENTING/STIMULATOR CELL SUSPENSIONS

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Although normal unfractionated spleen cell populations can be used as a source of accessory cells, in certain types of experiments it may be preferable to use spleen cell populations from which the T cells have been removed. This procedure ensures that none of the observed proliferative responses of the responder population result from T cell factors derived from the accessory cell population. For example, even T cells whose cell division has been blocked (second support protocol) can produce cytokines. In the following steps, T cell-depleted spleen cell suspensions are prepared using a lytic monoclonal antibody to the T cell antigen, Thy-1. Because almost all the antigen presentation or stimulator cell activity in spleen resides in the non-T cell fraction, this procedure also leads to enrichment of functional antigen-presenting cell function. Further enrichment of antigen-presenting cells (APC) by flotation of the T cell-depleted spleen cells on Percoll gradients is also described. Other procedures leading to enrichment of APC are described elsewhere; the method described in *UNIT 3.7* does not deplete T cells and therefore is not recommended here; the method described in *UNIT 3.15* leads to higher levels of enrichment that are not required in the protocols presented here.

Additional Materials

Spleen cells from nonimmunized mice

Hanks balanced salt solution (HBSS; *APPENDIX 2*)

Low-Tox rabbit complement (Cedarlane #CL3051), reconstituted with ice-cold distilled water and filter-sterilized

Anti-Thy-1.2 ascites (HO-13-4; ATCC #TIB 99) or anti-Thy-1.1 ascites (HO-22-1; ATCC #TIB 100; alternatively, see Table 3.4.1 for other anti-Thy-1 MAbs and *UNIT 2.6* for production of ascites)

70% Percoll solution (*UNIT 3.8* and reagents and solutions)

1. Centrifuge the spleen cell suspension derived from single spleen down to a pellet.

The spleen cells should always be from nonprimed animals and should be syngeneic to the responder T cells unless they are to be used as stimulator cells in the MLC.

2. To the pellet, add 0.9 ml HBSS, 0.1 ml complement, and 25 µl anti-Thy-1 ascites.

If cells from more than a single spleen are needed, the procedure should be scaled up accordingly.

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The choice of anti-Thy-1 reagent to be used depends on the strain of animal from which the spleen was derived. The great majority of commonly available mouse strains (except AKR) express the Thy-1.2 allele.

3. Incubate the mixture at 45 min in a 37°C water bath.
4. Centrifuge 10 min in Sorvall H-1000B rotor at ~1000 rpm ($200 \times g$), room temperature, and discard supernatant. Resuspend pellet in HBSS and wash two more times.
5. Count viable cells (APPENDIX 3) and resuspend in complete RPMI-10 or PBS for inactivation as in the second support protocol, or in HBSS to prepare low-density accessory cells (see below).

The T cell-depleted spleen cell population is comprised of B cells, macrophages, and dendritic cells. Further enrichment of cells with enhanced accessory cell function can be obtained by fractionation of this population on Percoll.

6. Dilute 70% Percoll solution to 55% by mixing 23.58 ml of the 70% Percoll with 6.42 ml HBSS. Resuspend T cell-depleted spleen cells from step 5 in HBSS at 20×10^6 cells/ml.
7. Layer 3 ml cell suspension over 3 ml of 55% Percoll solution in a 15-ml conical centrifuge tube.
8. Spin 13 min in H-1000B rotor at 3000 rpm ($1900 \times g$), room temperature.
9. Remove cells that band at the Percoll/HBSS interface with a 5-in. Pasteur pipet and wash 3 times in HBSS as in step 4.
10. Count viable cells and resuspend in complete RPMI-10 for inactivation according to the second support protocol.

The population obtained from steps 6 to 10 is comprised of large cells including macrophages, dendritic cells, and activated B lymphocytes. This population of cells is enriched in accessory cell function. When used in either of the basic protocols with purified T responder cells, fewer of the Percoll-purified cells should be needed to provide accessory function.

SUPPORT PROTOCOL

BLOCKING CELLULAR DIVISION OF ACCESSORY/STIMULATOR CELLS

There are two situations in which inhibition of accessory or stimulator cell division should be blocked. When purified T cells rather than unfractionated lymphoid populations are used in the basic protocol, cultures are frequently supplemented with accessory cells syngeneic to the responder T cells. If accessory cell DNA synthesis is inhibited, one can then be certain that the resultant proliferative response is comprised entirely of responder T cells and does not contain a component of recruited B cell proliferation derived from the accessory cell populations. In the MLR, the stimulator cells are spleen cells from mice that differ from the responder cells in *H-2* and/or *Mls* gene expression (see APPENDIX 1, Tables A.1C.1 and A.1F.1) and they can also recognize alloantigens on the responder cells. This responsiveness of stimulator cells against responder cells in an MLR (so-called back-stimulation) must be prevented by blocking cellular division. This can be done by treatment of stimulator cells with mitomycin C (a DNA cross-linking reagent) or by γ irradiation. Many investigators prefer mitomycin C treatment when antigenic differences encoded for by *Mls* genes are to be measured, or when an irradiation source is not available. For more information on the loci encoding *Mls* genes, see Tables A.1F.2 and A.1F.3.

Mitomycin C Treatment

Additional Materials

Mitomycin C (Sigma #M-0503; store in dark)

1. In a 15-ml aluminum foil-wrapped tube, prepare a solution of mitomycin C in PBS at 0.5 mg/ml and filter sterilize.

Since mitomycin C is very light-sensitive, it is necessary to prepare a fresh stock solution each day for each experiment.

2. Prepare spleen cell suspension as described in steps 1 and 2 of the basic protocol at a concentration of 5×10^7 cells/ml in PBS.
3. Add mitomycin C to a final concentration of 50 $\mu\text{g}/\text{ml}$ (100 $\mu\text{l}/\text{ml}$ of cell suspension) and wrap the tube in aluminum foil. Incubate 20 min at 37°C.
4. Add an excess of complete RPMI-5 (i.e., fill tube with ~12 ml) and centrifuge 10 min in Sorvall H-1000B rotor at 1200 rpm (300 $\times g$). Discard supernatant and repeat washing procedure two more times.

Three washes are crucial, because any traces of mitomycin C left among the cells will reduce proliferative responses when the cells are added to an MLC.

5. Resuspend pellet in complete RPMI-10. Count cells with hemacytometer. Adjust to desired concentration as described in the annotation to step 6 of the basic protocol.

Irradiation Treatment

Prepare a spleen cell suspension as described in steps 1 to 3 of the basic protocol, at a final concentration of $5-10 \times 10^6$ cells/ml in complete RPMI-10. Using a source of ionizing irradiation (^{60}Co or ^{137}Cs γ -irradiator; e.g., Gammacell 1000, Nordion), deliver 1000 to 2000 rad of irradiation to the cells.

This dose range of irradiation is suitable for most immunologic applications employing spleen cell suspensions. However, antigen presentation by different spleen cells is differentially affected by irradiation (Ashwell et al., 1984): at low doses (500 to 1000 rad), antigen-presenting function of B cells is preserved; after doses of 1100 to 2000 rad, a substantial decline is observed; and doses >2000 rad abolish the participation of B cells as APC. Macrophages and dendritic cells, on the other hand, maintain antigen presentation through doses of 3000 rad. To ensure that B cells do not participate in the responses measured, some investigators prefer to use doses of 2000 rad. However, responsiveness to *Mls* antigens can best be measured with stimulator cells that received doses of <1000 rad, since B cells present *Mls* more effectively. Alternatively, *Mls* responsiveness can be measured after mitomycin C treatment of stimulator cells, since it also preserves the antigen-presentation function of B cells.

When transformed cell lines are used as antigen-presenting or accessory cells, higher doses must be used to ensure blockage of cell division. The appropriate dose will have to be determined empirically for each cell line, but is likely to be at least 5000 rad; some transformed cell lines require as much as 10,000 to 12,000 rad, and may be more sensitive to mitomycin C treatment.

ACTIVATION OF PRIMED T CELLS

Proliferative responses to viruses, protein antigens, minor transplantation antigens, and the male H-Y antigen require *in vivo* immunization followed by *in vitro* stimulation. Furthermore, enhanced proliferative responses to those antigens that will generate primary *in vitro* responses (i.e., MHC antigens) can be obtained by *in vivo* priming. Multiple immunizations usually elevate *in vitro* responses.

To immunize animals for *in vitro* secondary responses to soluble protein antigens or peptides, dissolve antigens and emulsify in complete Freunds adjuvant (UNIT 2.5). For strong responses by draining lymph node cells, immunize animals in a hind footpad. For

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strong responses by spleen cells, immunize intraperitoneally. Tail-base immunization also can be used as an efficient route of immunization; follow procedure for intradermal injection. To prime animals against cellular antigens, inject intraperitoneally with $1-5 \times 10^7$ cells that express the antigen. Immunization protocols are described in *UNIT 1.6*.

Within 2 to 3 weeks after in vivo priming, in vitro responsiveness of primed T cells can usually be measured. This assay is often used as a preparation for subsequent in vitro cloning procedures (*UNIT 3.14*) and T cell hybridoma preparation (*UNIT 3.13*).

Materials

Complete RPMI-10 medium (*APPENDIX 2*)

Responder cells: Purified T cells isolated from lymph nodes (*UNITS 3.1-3.6*) of in vivo primed mice

Antigen: 1 mg/ml sterile protein antigen(s) (*UNIT 3.13*), in PBS or suspension of irradiated or mitomycin C-treated stimulator cells expressing alloantigens at 8×10^6 cells/ml (*UNIT 3.11*, support protocol) in complete RPMI-10 medium (*APPENDIX 2*)

Accessory cells: suspension of irradiated or mitomycin C-treated (or T cell-depleted) spleen cells syngeneic to the responding T cells at 5×10^6 cells/ml in complete RPMI-10 medium

4-ml conical tubes

96-well flat-bottom microtiter plates with lids

1. Follow steps 1 to 3 of the first basic protocol for preparation of responder cells.
2. Prepare 4-fold dilution series of the antigens in 4-ml conical tubes, using complete RPMI-10.

The following dilutions are recommended: 100, 10, 1, and 0.1 µg/ml protein antigens and 8, 4, 2, and 1×10^6 cells/ml of stimulator cells in complete medium.

3. Add antigens to 96-well flat-bottom microtiter plates, at 30 µl/well for protein antigens or 100 µl/well for cellular antigens. For each experimental group, set up three replicate wells and include control wells with medium only (no antigen).

By using four concentrations of antigens and three replicate wells for each dose, one row of a microtiter plate will cover the entire tested range.

4. Add responder T cells in 0.1 ml to each well.

Purified T cells are recommended; otherwise extremely high background values may be obtained. This appears to be due in part to proliferation of recruited cells (T and non-T) that are not antigen-specific. If unfractionated lymph node cells from recently primed mice are used, add $1-2 \times 10^5$ cells per well and proceed to step 6.

5. If purified lymph node T cells specific for protein antigens are used, add 0.1 ml of accessory spleen cells syngeneic to the donor of the responder T cells at 5×10^5 cells per well.

Purified T cells require an exogenous source of accessory non-T cells. Accessory cells function both as antigen-presenting cells and as a source of undefined "second signals." They are not required for cell preparations primed against cellular antigens, because accessory cell function is provided by the stimulator cells.

6. Proceed as in steps 7 to 9 of the basic protocol.

Culture periods before labeling can vary widely and kinetic assays should be performed. In general, for T cells from primed mice, it is likely that the response will peak at day 4 or 5.

REAGENTS AND SOLUTIONS

Percoll solution

Diluent:

45 ml 10× PBS, pH 7.4 (APPENDIX 2)

3 ml 0.6 M HCl

132 ml H₂O

Filter sterilize

70% Percoll solution:

63 ml Percoll (Pharmacia LKB #170891-01)

37 ml sterile diluent (above)

Final osmolarity should be 310 to 320 osM

COMMENTARY

Background Information

Proliferative assays for measuring T cell function have certain advantages and disadvantages compared to the cytotoxic T lymphocyte (CTL) assay described in UNIT 3.11 or the lymphokine production assays in UNITS 3.15 & 6.3. Advantages are that proliferative assays are less time-consuming, less labor-intensive, less cell-consuming, and less expensive than "true" effector T cell function assays. A disadvantage is that antigen specificity is not as easily demonstrated in proliferative assays as in CTL assays, unless antigen-specific clones of proliferating cells are used. Furthermore, the proliferative assay only detects dividing cells instead of measuring true effector T cell function.

It is not clear which T cell function is measured in proliferative assays; the proliferative response should therefore be used solely as general indicators of T cell reactivity. Data obtained in proliferative assays might variably reflect proliferation of CTL, lymphokine-producing T cells, or nonactivated "by-stander" cells, and will be severely affected by the function of non-T cells such as accessory cells (see below). Since the majority of T cells respond to and produce IL-2 upon activation, differences in responsiveness in a proliferative assay in part reflect differences in IL-2 production by the responding T cells. Proliferative assays therefore become more meaningful when combined with the lymphokine detection assays presented in UNITS 3.15 & 6.3. Since responsiveness to IL-2 is also determined by the levels and functionality of IL-2 receptors, further information will be added by including measurements of IL-2 receptors (UNIT 6.1) or by flow cytometry (UNIT 5.4). Yet, as a first approximation of cellular activation, proliferative assays are valuable.

Critical Parameters

and Troubleshooting

Parameters affecting the magnitude of T cell proliferative responses include cell concentration, type of medium, source of serum, incubator conditions (CO₂ level and humidity), type and concentration of activating agent, type of responding T cells, type of accessory/stimulator cells, mouse strain, and culture time. Optimal conditions for individual laboratories and experiments must be derived empirically with respect to these variables, but general guidelines are provided below.

A number of agents can be employed in the first basic protocol to induce T cell proliferation (Table 3.12.1). T cells may be activated by pharmacologic means by producing an elevation of intracellular free calcium with a calcium ionophore combined with activation of protein kinase C with a phorbol ester. The most direct means of inducing T cell activation involves stimulation with monoclonal antibodies that interact with the CD3/TCR complex—i.e., anti-CD3, anti-TCR- $\alpha\beta$ or $\gamma\delta$, as well as anti-V β antibodies that are capable of interacting with a subset of cells bearing a specific TCR. A vigorous T cell proliferative response of defined subsets can also be induced with certain bacterial toxins known as staphylococcal enterotoxins. These toxins are often referred to as "superantigens" (Marrack and Kappler, 1989) because they stimulate T cells via the variable (V) gene segment of the TCR. Different toxins have affinities for different V β chains and these specificities make them valuable reagents for activating T cells. The activating capacity of toxins is also dependent on their ability to bind to MHC class II molecules (i.e., responding T cells react with the toxin/class II complex); thus, responsiveness varies with the

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mouse strain used. Lectins such as phytohemagglutinin (PHA) and concanavalin A (Con A) have been widely used for many years to activate T cells. Although the precise mechanism of action of these agents is unknown, it is likely that lectins activate T cells by indirectly cross-linking the TCR because TCR-negative cells will not respond to these agents. Lastly, it is also possible to induce T cell activation with monoclonal antibodies to cell-surface antigens other than the TCR; this protocol employs the G7 monoclonal antibody, one of the most effective of the anti-Thy-1 activators (Gunter et al., 1984).

When comparing the reactivity of different cell populations, it is essential to perform dose-response assays for responder T cells and activating agents and for both responder and stimulator T cells (in MLR), since each population may yield optimal responses at different cell numbers. This may reflect differences in frequency of responding cells, and hence may indicate a need to perform limiting dilution assays (*UNIT 3.15*). Since peak responsiveness of different populations of T cells may occur at different times, it is also essential to perform kinetic experiments—i.e., compare responsiveness at days 2, 3, 4, and 5.

Differences in responsiveness need not necessarily be due to differences in the frequency of responding T cells, but may also indicate differences in the efficacy with which co-stimulatory activity or “second signals” are delivered by the accessory cells present in different cell populations. The type of interactions pertinent to the generation of primary responses by T cells is explained in the commentaries of *UNITS 3.8, 3.11, & 3.13*. Specific requirements for inducing activation with immobilized antibodies have been described (Staerz and Bevan, 1986; Hathcock et al., 1989; Jenkins et al., 1990). A responding cell population completely devoid of accessory cells (such as purified populations of splenic or lymph node T cells or cloned T cells) will yield fine responsiveness in an MLC, since accessory cell function is provided by the stimulator cells; however, the same population will generally not yield responses when mitogens, antigens, or enterotoxins are used. In such a setting, accessory cells may also function as antigen-presenting cells (APC). Addition of irradiated or mitomycin C-treated syngeneic sources of accessory cells (either whole spleen cells or purified APC; see first support protocol) can be used to restore responsiveness in purified T cells. The need for accessory cells can sometimes be

bypassed when anti-TCR monoclonal antibodies are coupled to plastic, or when certain anti-Thy-1 monoclonal antibodies are used; however, these conditions do not necessarily result in optimal responsiveness (Jenkins et al., 1990).

The level of [³H]thymidine incorporation should not be regarded only as a reflection of cellular proliferation: some nondividing cells will synthesize DNA and “cold” thymidine released by disintegrating cells will compete with incorporation of labeled thymidine. Therefore, measurements of DNA synthesis should be accompanied by counting viable cells over the length of the culture period if a true estimate of cellular proliferation is to be obtained. Of course, cell death of nonactivated cells will also interfere with the accuracy of this last parameter.

The sensitivity of proliferation assays is such that small errors in cell numbers will result in large differences in [³H]thymidine incorporation values. When values obtained in triplicate cultures correspond poorly (e.g., >5% difference in cpm values >1000), technical problems such as cell clumping, dilution, and pipetting should be considered. Excessively high values may be obtained from contaminated wells, as [³H]thymidine will be incorporated into replicating bacteria; therefore, it is good practice to check the wells from microtiter plates under an inverted microscope for contamination. Contamination may also interfere with proliferation of the activated lymphocytes.

It is also useful to check for blast formation by microscopic examination of the cultures: activated lymphocytes will tend to enlarge, and detection of blasts will give a general indication of successful activation.

The main problem that may occur with proliferative response assays is high levels of background [³H]thymidine incorporation in control cultures without antigens. This problem is frequently due to the fetal calf serum (FCS) used to supplement the cultures, which may be mitogenic for B cells. Different lots of FCS should be screened to select those that are nonstimulatory or only weakly stimulatory in the absence of other stimuli, and that support strong proliferative responses upon antigenic stimulation of T cells.

If flat-bottom microtiter plates are used in the procedure and weak responses occur, it may be useful to switch to round-bottom plates. Our laboratory has found consistently better responses in round-bottom plates when

thymocytes are used as responder cells or with slight alloantigenic differences between responding and stimulating cells. In addition, antibody-mediated experiments yield better results with round-bottom plates. Presumably, this reflects better cell contact obtained in such plates; optimal responses will almost certainly occur at different cell numbers than in flat-bottom plates and densities will have to be adjusted accordingly.

Although satisfactory responses to most alloantigens can be obtained with complete RPMI-10 medium, it may be necessary to compare different media. This need arises when the proliferative responses are weak (i.e., when [³H]thymidine values for activated cultures are <10-fold higher than those for control cultures) and may occur under various circumstances: weak alloantigenic differences between responder and stimulator cells, weak T cell proliferative function in the responder cells or diminished APC function in the stimulator cells due to experimental manipulations, or a low precursor frequency of responding T cells. Thymocytes in particular do not contain a high level of responding T cells. Frequently, proliferation can be improved when complete Clicks or Dulbeccos media are used (with additives as described in APPENDIX 2), presumably because these media contain additional nutrients and have an osmolarity more compatible with mouse serum than RPMI.

When RPMI is used as medium, 5% CO₂ will be sufficient, but for other media, a 7.5% CO₂ concentration in the incubator will be more satisfactory. Generally, the buffering capacity of DMEM is insufficient at 5%, but fine at 7.5%. Much will also depend on the proliferative activity of the responding population of T cells (e.g., vigorous proliferation will reduce the pH in the cultures); it is therefore recommended to compare responsiveness in initial pilot experiments in incubators set at different CO₂ concentrations.

The culture period required for stimulation—after which the cells are to be labeled—varies for different laboratories, media, and types of responding and stimulator cells. Conditions eliciting weak responses, such as those obtained with thymocytes or a weak alloantigenic difference, will require a longer culture period (5 to 6 days) than those which elicit a higher frequency of responding T cells (3 to 4 days). Because laboratory conditions vary, it will be necessary to run a kinetic assay to determine the optimal time for T cell prolifer-

ation. Addition of [³H] thymidine on days 2, 3, 4, 5, and 6 will provide a useful test; further extension of the culture period will not yield any improvements, due to exhaustion of nutrients in the medium.

Anticipated Results

For proliferative assays described in the basic protocol, which activate the majority of the responding T cells, responses of 100,000 cpm should be obtained; in the MLR or following activation with monoclonal antibodies to subpopulations of T cells (anti-V β), responses up to 100,000 cpm may be observed; however, measurements of 20,000 cpm (with tight standard errors) can be quite satisfactory. Background values of <1000 cpm should be expected. Reported results (as described in step 9a) should be mean cpm of experimental wells minus background cpm (Δ cpm).

Time Considerations

The time required to set up proliferative assays is not more than a day, with the number of hours depending on the number of different groups of responder cells that must be prepared. The time required for incubation of cells ranges from 2 to 6 days, as noted above in critical parameters. Following an additional 18- to 24-hr incubation period for pulsing, harvesting the cells and measuring cpm will require several hours depending on the number of plates (~15 min for harvesting each plate and ~100 min for counting each plate at 1 min/sample).

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In Vitro Assays
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Both of the above describe how specific proliferation of antigen-primed T cells can be measured.

Details the MLC proliferation assay.

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Dendritic cells are allowing scientists to overcome a longstanding obstacle to research in immunology by extending the playing field beyond antigens to immunogens and beyond models to pathogens that cause disease.

The Dendritic Cell Advantage: New Focus For Immune-Based Therapies

by Ralph M. Steinman

The focus of immune therapeutics has been on lymphocytes, the cellular mediators of immunity, and the suppression of lymphocyte function. The drug cyclosporin (cyclosporine) is an excellent and successful example. However, medicine needs therapies that enhance immunity or resistance to infections and tumors. Medicine also needs strategies, whether suppressive or enhancing, that are specific to the disease-causing stimulus or antigen. In contrast to lymphocytes, dendritic cells (DCs) provide a much earlier and antigen-specific means for manipulating the immune response. DCs capture antigens and then initiate and control the activities of lymphocytes, including the development of resistance to infections and tumors (reviewed in references 1-3).

Summary

Dendritic cells (DCs) provide a much earlier and antigen-specific means for manipulating the immune response. The best-studied function of DCs is to convert antigens into immunogens for T cells. The "DC advantage" entails a myriad of functions. DCs are more than antigen-presenting cells; they are accessories or adjuvants or catalysts for triggering and controlling immunity. Another special feature of DCs is their location and movement in the body; DCs are stationed at surfaces where antigens gain access to the body. The events that make up the life history of DCs are now being unraveled in molecular terms. As research on DCs expands, more potential functions and more sites for their manipulation are becoming apparent. © 2000 Prous Science. All rights reserved.

The controlling role of DCs is best known for thymus-dependent lymphocytes or T cells which are important in many diseases, the most poignant being the AIDS epidemic (Table 1). DCs were identified in a few laboratories that were focusing on the induction of immunity from resting T cells. It was noted that immune tissues (spleen, lymph nodes, lymph, blood) had a small fraction of cells with unusual

"tree-like" or "dendritic" processes. These distinctive cells had not been recognized previously and they proved to have distinct functions. Most importantly, DCs were potent inducers of immunity even in animals, not just the test tube, and now even in patients (reviewed in references 1-3).

The DC field was held back by the fact that there were so few cells relative

TABLE I: HUMAN DISEASES THAT INVOLVE T CELLS

- Rejection of organ transplants and graft-vs.-host disease in bone marrow transplantation
- Resistance to many infections including vaccine design
- Vaccines against tumors and immune therapies for existing tumors
- Allergy
- AIDS
- Autoimmune diseases like insulin-dependent (juvenile) diabetes, multiple sclerosis, rheumatoid arthritis and psoriasis

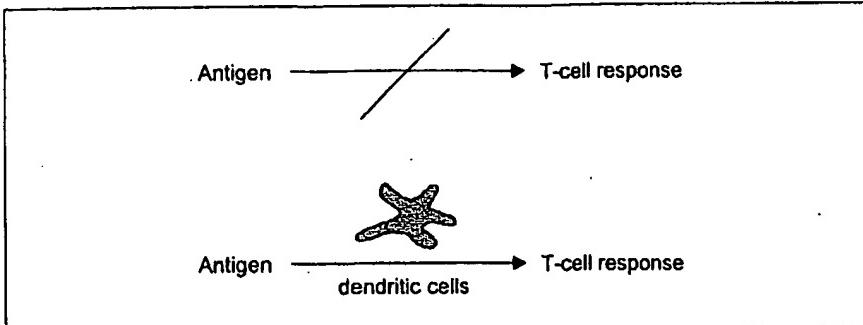


Fig. 1. A key function of dendritic cells. Antigens within tumors, transplants and infectious agents need to be presented by DCs to become immunogens, i.e., to make T cells begin to grow and exhibit their helper and killer functions.

to other players in the immune system such as B cells, T cells and macrophages. In reality, DCs are quite abundant for the job they have to do, namely, to initiate immune responses from antigen-specific T cells. In immune system organs like lymph nodes, DCs form an extensive network throughout the T cell-rich regions and physically outnumber any given antigen-reactive T cell by at least 100 to 1. The DC field was also held back because many thought that the cells were no different from macrophages, thus keeping investigators from working on the active DCs. In reality, DCs were identified on the basis of profound differences from macrophages, and their many distinct properties and functions were only uncovered by separating DCs from macrophages.

The best-studied function of DCs is to convert antigens into immunogens for T cells. The antigen receptors on T cells do not focus on intact proteins in microbes and tumors, but instead recognize fragmented or processed proteins, that is, peptides. The processing of protein antigens into peptides occurs within cells, and then the peptides are

displayed or presented at the cell surface affixed to products of the major histocompatibility complex (MHC). The ensuing interaction between a T-cell receptor (TCR) and its specific MHC-peptide complex allows a T cell to detect peptides formed within cells in transplants, tumors, sites of infection and self tissues attacked during autoimmune disease (Table I). "Antigens" refers to specific substances recognized by the immune system, while "immunogens" refers to antigens that effectively induce responses either by themselves or together with enhancing materials called "adjuvants." For T cells in particular, antigens and immunogens are not one and the same (Fig. 1). Even preprocessed peptides and MHC-peptide complexes are weak immunogens. This was evident early on in the work of Peter Medawar, the great scientist who discovered the immune basis of transplantation. He spent many years trying to purify functioning transplantation antigens. These efforts were to little avail.

What was not known in Medawar's time is that transplantation antigens

(later shown to be MHC-peptide complexes) become immunogenic when presented by DCs.⁴ In other words, transplantation antigens when presented on many cell types are weak immunogens, but on DCs they become powerful inducers of immunity.⁴ The same is true of peptides that become much more immunogenic when presented on DCs. DCs activate T cells by getting them to divide and express their helper and killer functions. Then the activated T cells interact with other antigen-presenting cells to eliminate the antigen in question. DCs are also called "nature's adjuvant," because prior adjuvants were artificial substances used to enhance immunity. The DC advantage entails a myriad of functions, some of which will be considered below.

Potency of dendritic cells in initiating immunity in tissue culture

What are some specific features of DCs that warrant attention? The first is their potency. Very small numbers of DCs are sufficient to trigger strong T-cell responses in test tubes. Immune assays are generally carried out with impure antigen-presenting cells, applied at a dose of one presenting cell for every T cell, the latter often preactivated. In contrast, roughly one DC per 30–100 T cells is more than sufficient to induce optimal responses, including responses by resting T cells. A single DC can simultaneously activate 10–20 T cells nestled within its sheet-like processes. Therefore, DCs are more than antigen-presenting cells; they are accessories or adjuvants or catalysts for triggering and controlling immunity.

It has always been clear that the accessory function of DCs did not depend exclusively on their capacity to process antigens to form MHC-peptide complexes. This is because the stimuli that were used to define the potency and immune-activating role of DCs did not require that the DCs process an applied antigen. Such stimuli included major transplantation antigens, mitogens, contact allergens, anti-

T-cell antibodies and superantigens. Furthermore, once resting T cells were activated by DCs, the T cells responded vigorously to antigens presented by other cell types, showing that the latter were not deficient in forming ligands for the antigen receptor on T cells, but instead lacked accessory properties.

The word "accessory" has since been replaced by the terms "professional" and "co-stimulatory," but the basic concept is unchanged by shifting terminology. T cells need stimuli other than their specific trigger or ligand (MHC-peptide complexes) to begin to grow and function, for example, to produce the interleukins and killer molecules mentioned above. DCs are potent in providing the needed accessory or co-stimulatory functions. For example, DCs produce an adhesion molecule called DC-SIGN that binds to a target on resting T cells called ICAM-3,⁵ and DCs express very high levels of a stimulatory molecule called CD86 that binds to CD28 on resting T cells.⁶ These are but two examples of the specialized activities of DCs. These cells do not operate as a single magic bullet.

Position of dendritic cells *in vivo*

Another special feature of DCs is their location and movement in the body. As criteria were developed to identify DCs, it became feasible to go back into the animal and patient to look for the corresponding cells in different tissues. DCs are stationed at surfaces where antigens gain access to the body (Fig. 2, left). The skin and the airway have been the best studied. DCs are found in afferent lymphatic vessels, special channels that allow cells to move from peripheral tissues to lymphoid organs, primarily the T-cell areas (Fig. 2, middle and right). This migration is most readily observed in models of skin transplantation and contact allergy, which are the two most powerful immune responses known.

DC migration is likely to be very important. The body's pool of T cells primarily traffics through the T-cell areas of lymph nodes, rather than

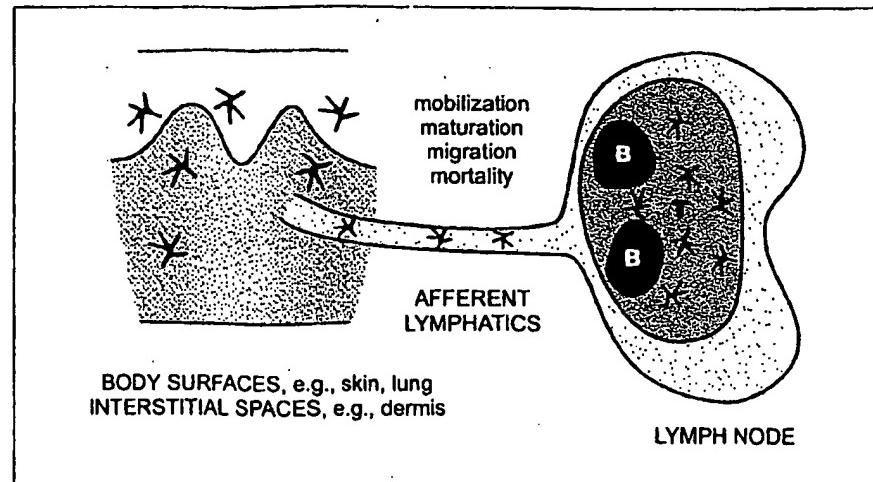


Fig. 2. Distribution of dendritic cells *in situ*. DCs at body surfaces and in solid organs can pick up antigens, move to the lymphoid tissues to find antigen-specific T cells and initiate immunity. Molecular mechanisms are being uncovered that govern the mobilization, maturation, migration and mortality of these DCs. In the lymph node, T lymphocytes are selected for expansion and differentiation into helper and killer T cells. The activated T cells then leave the lymph node to return to the body surface or peripheral organ to eliminate the antigen.

through tissues where antigens are usually deposited. So when DCs capture antigens in the skin, airway or another peripheral tissue, their migration to the T-cell areas gives them a chance to select the corresponding rare specific T cells from the assembled repertoire (Fig. 2). The selected T cells then increase in numbers (clonal expansion) and function, enabling the specific immune response to begin. The initial frequency of T cells that recognize an antigen is very small. Only one in 10,000–100,000 of T cells in the repertoire responds to a specific MHC-peptide complex. Therefore, it is so precise and efficient for DCs to be able to pick up an antigen in the periphery and then initiate the immune response from rare T-cell clones in lymphoid organs.

The events that make up the life history of DCs (Figs. 2 and 3) are now being unraveled in molecular terms. For example, scientists are figuring out how to expand antigen-capturing precursors to DCs using IL13 ligand and granulocyte colony-stimulating factor (G-CSF). Key players for the mobilization of DCs from the periphery to lymph nodes are the multidrug resistance receptors, usually studied for their capacity to mediate resistance to chemotherapeutic agents rather than

movement of DCs. Migration of DCs is controlled by chemokines produced in the lymphatic vessels and lymphoid organs (Fig. 2). These act on DC chemokine receptors to orchestrate their movement to the T-cell areas. Then within the lymphoid tissue, several members of the tumor necrosis factor (TNF) and TNF-receptor families, such as TRANCE and CD40 ligand, trigger DC production of cytokines like interleukin-12. The TNF family also maintains DC viability. Otherwise the cells die within a day or two. Each of these components of DC function provides targets for manipulating immunity.

Priming of T-cell immunity via dendritic cells

Animal studies

During the early research on DCs, several labs administered antigens to experimental animals and then tried to identify the cells that had captured the antigens in a form that was immunogenic. Regardless of the route of antigen administration (blood, muscle, skin, intestine and airway), DCs were the major reservoir of immunogen.

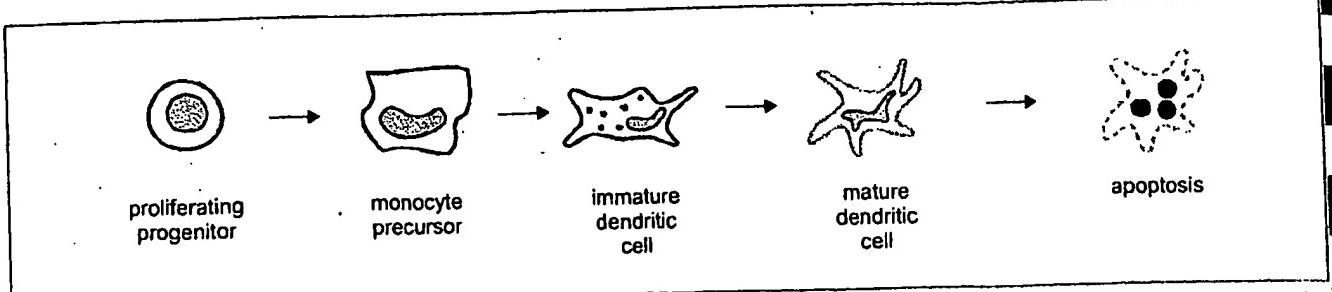


Fig. 3. The life history of dendritic cells. DCs arise from proliferating progenitors, primarily in the bone marrow, and this can be driven by cytokines like flt-3 ligand and G-CSF. Precursors are formed, such as the monocytes in blood, which then give rise to immature DCs. The immature DCs are capable of producing large amounts of antigen-presenting MHC products and capturing antigens. Multidrug resistance receptors are newly recognized players in the mobilization of immature DCs. DCs mature in response to various stimuli such as infection and inflammation, and migrate under the influence of chemokines to the lymphoid tissues. There the DCs die within a day unless their lifespan is prolonged by TNF-family molecules expressed by the activated T cells.

Next, DCs were used as nature's adjuvant to immunize animals. The DCs were taken from mice or rats, exposed to antigens *ex vivo* and injected back into immunologically naive recipients. The animals became immunized to the antigens that had been captured by the DCs, and the immunization took place in the lymph nodes draining the site of DC injection. Genetic proof was provided that the DCs were priming the animal directly and not simply handing off their antigen to other cells.^{7,8}

DC-based immunization is really very different from all prior attempts at cell therapy. Immunology has had extensive experience with "passive immunization," whereby a recipient is given large numbers of cells that are activated prior to injection. It is hard to produce such large numbers of cells, and their lifespan, diversity and efficacy are all finite. In contrast, when relatively small numbers of antigen-charged DCs are used to induce immunity, this produces "active immunization." Now the animals (and patients, see below) can make their own diverse and longer lasting immune response to the antigen-bearing DCs.

Human studies

The above experiments made it clear that DCs, pulsed *ex vivo* with antigens, actively immunized animals and raised the exciting possibility that scientists would be able to induce resis-

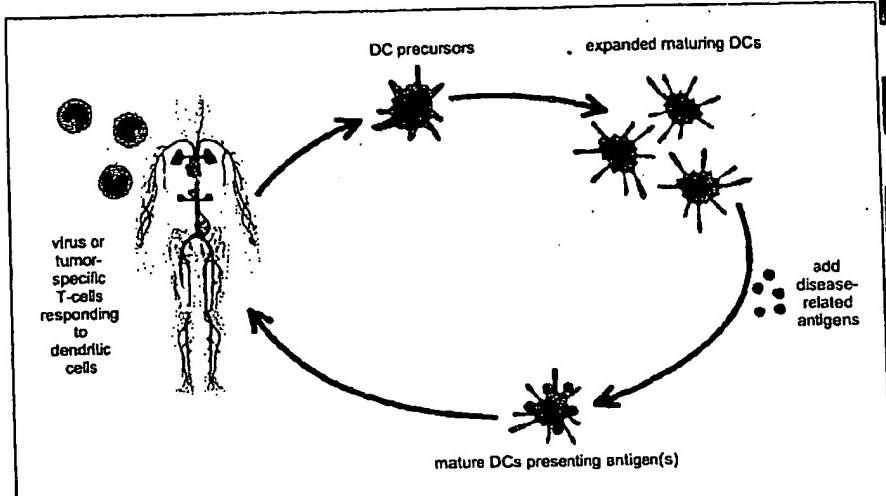


Fig. 4. The use of dendritic cells as adjuvants for enhancing immunity to tumors and infectious agents in humans. This new form of immune therapy begins with the isolation of DC precursors from the patient, usually from blood. The precursors develop *ex vivo* (in relatively simple tissue culture systems) into large numbers of more mature DCs. During this time, the DCs are charged with antigens from the tumor or infection. Then the DCs are reinfused to elicit immunity and thereby resistance to the disease.

tance to tumors, infections and transplants in patients. For example, could one expose patients' DCs *ex vivo* to antigens in their tumors and then reinject the antigen-bearing DCs to elicit tumor-specific immunity (Fig. 4)? This approach is actually not terribly complicated, but one first had to overcome a major obstacle and learn to generate large numbers of DCs. These techniques became available in the 1990s. They have energized the field and, accordingly, clinical trials for the immunization of humans against cancer have begun on most continents.

It is evident that DCs can serve as adjuvants for humans, converting antigens into immunogens.^{9,10} Even in advanced cancer, immune responses already have been observed that are similar to or better than immune responses obtained with other approaches. However, this approach is still in its preliminary stages, since a good deal of science remains to be developed. On the one hand, there are critical unknowns in terms of overall DC biology. Many of the clinical studies to date, for example, have overlooked key features that could improve DC function, such as the need for DCs

to be sufficiently mature (see below) to be effective *in vivo*. Also, DC biology has to be placed in the context of specific tumors and pathogens and patients for DC-based therapies to be optimized.

To summarize and further illustrate the role of DCs in the context of human disease (Table I), consider the need to harness T cells to resist tumors and chronic infections. Protein antigens often are known for a tumor-like melanoma, or for a virus like HIV-1 whose genetic sequence has been available for more than 15 years. However, this knowledge about antigens from melanoma and HIV-1 antigens remains to be converted into methods that provide better immunogens either for immune therapy of melanoma or for the design of HIV-1 vaccines. This is because some important facts of immunological life are being overlooked. When antigens are injected, they also need to gain access to the right DCs to become immunogens (Fig. 1).

Delivering antigens to dendritic cells

Broadly speaking, a central goal is to learn how to deliver or "target" antigens to DCs and simultaneously to differentiate or "mature" the cells to their most potent state. These two challenges, antigen targeting and DC maturation, prove to be intertwined.

Targeting means that the antigen should be in a form that the DCs can recognize. Without such recognition, the uptake and subsequent processing of antigen to form MHC-peptide complexes is suboptimal. DCs have a number of special mechanisms for capturing antigens and converting these into MHC-peptide complexes (Table II). For example, DCs have a receptor called DEC-205 whose binding partners or ligands are still unknown. Nonetheless, it is clear that DEC-205 greatly increases the capacity of DCs to form MHC-peptide complexes.¹¹ DCs also carry out a fascinating process called "cross-presentation." DCs can take up dying cells and effi-

TABLE II: DENDRITIC CELL SPECIALIZATION TO INCREASE MHC-PEPTIDE COMPLEX FORMATION

- Receptors for antigen uptake, e.g., DEC-205
- Processing of dying cells, nonreplicating microbes and immune complexes onto MHC class I ("cross-presentation")
- Regulation of antigen processing by maturation stimuli
- Clustering of T-cell receptor ligands with co-stimulators like CD86

ciently extract peptides from them, so antigens "cross" from the dying cell to the DC. The discoverers of this phenomenon called it "resurrecting the dead."¹² Cross-presentation allows DCs to efficiently form MHC-peptide complexes from dead cells in tumors, transplants and tissues under autoimmune attack.

Special uptake and processing mechanisms allow DCs to tailor a protein antigen, as well as the proteins in a complex microbe or tumor cell, into peptides that bind to an individual's MHC products. The latter are exceptionally polymorphic, differing genetically from one individual to another. As a result, the relevant immunizing peptides differ from one individual to another. One reason why peptides are not ideal immunogens is that they must be individualized. DCs, in contrast, can capture antigens with high efficiency and likewise extract peptides that are relevant for any individual.

A second DC advantage is that these cells have the many required accessory or co-stimulatory properties for converting the selected peptides ("antigens") into effective immunogens. A third DC advantage is that these cells position themselves in a way that leads to the identification of rare antigen-reactive T lymphocytes *in vivo* (Fig. 2). DCs thus overcome many of the difficult obstacles in initiating immunity.

In order for an antigen to be a strong immunogen, one needs to provide a stimulus for the final differentiation or maturation of the DCs (Fig. 3). Most DCs in the body are in an immature state and lack many features that lead to a strong T-cell response.

Immature DCs, for example, lack the CD86 and CD40 molecules that greatly boost the DC-T cell interaction. Immature DCs also lack a chemokine receptor called CCR7 that seems very important for proper migration and homing to lymph nodes to start immunity. For cancer immunology, it is unlikely that tumors provide maturation stimuli. Tumors may even block DC maturation induced by other stimuli. Therefore it is important to learn how to deliver tumor cells to DCs and bypass the normal obstacles to effective antitumor immunity.

Surprising recent evidence actually links DC maturation to the efficient formation of MHC-peptide complexes or TCR ligands (Table II). Immature DCs take up antigens, but they do not make abundant MHC-peptide complexes until they receive a maturation stimulus.^{13,14} Maturation also up-regulates CD86 co-stimulators, but the CD86 actually travels together with the TCR ligands to the surface of the DCs. At the DC surface, the MHC molecules and CD86 remain clustered with each other, keeping the machinery for T-cell activation juxtaposed. This phenomenon will help explain the potency of DCs, because TCR ligands and co-stimulators are displayed together on the cell surface and in high levels.

Control points beyond antigen targeting and maturation of DCs

Research on DCs is moving more vigorously, because the cells are more readily available and because their role in the immune system is considered essential. Nonetheless, researchers in this field are just beginning to find ways to manipulate DCs *in situ*. Putting together an antigen that targets

to DCs with a stimulus for DC maturation will be a major step in improving the conversion of antigens into immunogens, as in immune-based therapies against tumors and infectious agents.

Additional challenges and questions are evident:

- How can DC numbers be increased *in situ* and how can active DCs be mobilized to a cancer or site of chronic infection?
- Can DCs induce strong immune memory to make vaccination long lasting and effective (we have only been reviewing the role of DCs in the initiation of immunity)?
- Can DCs change the quality of the immune response? "Quality" refers to recent evidence for different types of DCs, especially a subset that induces Th1-type T cells for resistance to infectious agents and strong memory.
- Is it possible to move beyond DC-based immunization experiments and use DCs to either regulate or tolerize the immune system, as frequently required in transplantation and autoimmune diseases?
- Can DCs influence elements of the immune system other than T cells; for example, B cells and the innate defenses provided by natural killer (NK) and NK-T cells?

The answer to all these questions is a preliminary "yes." As research on DCs expands, more potential functions and more sites for their manipulation are becoming apparent.

Dendritic cells and better control of disease

DCs provide important avenues for the investigation of human disease. Many labs are exploiting DCs to identify antigens relevant for immunity against human pathogens. In these experiments, one introduces complex but clinically important antigens to DCs and then identifies which components are best presented to the immune system. We have recently used this approach to identify previously un-

known immune responses to the Epstein-Barr virus,¹⁵ a virus we all carry that has the potential to cause cancer like Hodgkin's lymphoma. Other laboratories have been using DCs to identify new antigens in other infectious agents, in transplants and in cancers like melanoma.

Investigators are also manipulating DCs *ex vivo* and then reinfusing the cells to identify conditions leading to strong immunity in patients (Fig. 4). In particular, DC-mediated active immunization against cancer is being vigorously pursued, as mentioned above. Instead of manipulating DCs *ex vivo*, a more desirable goal would be able to alter DCs directly *in situ*. Some approaches are under way. An example is the injection of cytokines like IL-3 ligand and G-CSF to mobilize various precursor populations of DCs. One should also develop methods to control DC mobilization, migration and maturation. In sum, DCs are allowing scientists to overcome a longstanding obstacle to research in immunology by extending the playing field beyond antigens to immunogens and beyond models to pathogens that cause disease.

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Coexpression of two distinct genes is required to generate secreted bioactive cytotoxic lymphocyte maturation factor

(heterodimeric lymphokine/T-cell growth factor/lymphokine-activated killer cells/coordinate gene regulation/interleukin-12)

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ABSTRACT Cytotoxic lymphocyte maturation factor (CLMF) is a disulfide-bonded heterodimeric lymphokine that (i) acts as a growth factor for activated T cells independent of interleukin 2 and (ii) synergizes with suboptimal concentrations of interleukin 2 to induce lymphokine-activated killer cells. We now report the cloning and expression of both human CLMF subunit cDNAs from a lymphoblastoid B-cell line, NC-37. The two subunits represent two distinct and unrelated gene products whose mRNAs are coordinately induced upon activation of NC-37 cells. Coexpression of the two subunit cDNAs in COS cells is necessary for the secretion of biologically active CLMF; COS cells transfected with either subunit cDNA alone do not secrete bioactive CLMF. Recombinant CLMF expressed in mammalian cells displays biologic activities essentially identical to natural CLMF, and its activities can be neutralized by monoclonal antibodies prepared against natural CLMF. Since this heterodimeric protein displays the properties of an interleukin, we propose that CLMF be given the designation interleukin 12.

The molecular cloning and expression of recombinant cytokines has made possible both significant advances in our understanding of the molecular basis of immune responses and the development of new approaches to the treatment of disease states. As an example, recombinant interleukin 2 (recombinant IL-2) has been shown to be capable of causing regression of established tumors in both experimental animals (1) and in man (2); however, its clinical use has been associated with significant toxicity (2). One potential approach to improving the therapeutic utility of recombinant cytokines is to use them in combination (3, 4). With this concept in mind, we initiated a search for novel cytokines that would synergize with suboptimal concentrations of recombinant IL-2 to activate cytotoxic lymphocytes *in vitro* and thus might have synergistic immunoenhancing effects when administered together with recombinant IL-2 *in vivo*. This led to the identification of a factor, designated cytotoxic lymphocyte maturation factor (CLMF), that synergized with recombinant IL-2 to facilitate the generation of both cytolytic T lymphocytes (CTLs) and lymphokine-activated killer (LAK) cells *in vitro* (5, 6). CLMF was subsequently purified to homogeneity from a human lymphoblastoid B-cell line (NC-37) and was shown to be a 75-kDa disulfide-bonded heterodimer composed of two subunits with molecular masses of 40 kDa and 35 kDa (7).¹ We now report the molecular cloning and expression of CLMF.

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MATERIALS AND METHODS

cDNA Cloning. A subline of NC-37 cells selected for its ability to produce high levels of CLMF (7), NC-37.98, was induced with phorbol 12-myristate 13-acetate (PMA) and calcium ionophore A23187 for 16 hr. Poly(A)⁺ RNA was isolated, and random hexamer-primed cDNA libraries were established in phage λgt10 by standard procedures. Mixed-primer polymerase chain reaction (PCR) using controlled ramp times (8) was performed as follows. PCR primers contained all possible codons and were 14 or 15 nucleotides long (Fig. 1) with a 5' extension of 9 nucleotides containing an EcoRI site for subcloning. Degeneracies varied from 1 in 32 to 1 in 4096; 0.5–4 pmol per permutation of forward and reverse primer was used in a 50- to 100-μl PCR mixture with 40 ng of cDNA made from NC-37.98 cells that had been activated by culture with 10 ng of PMA and 25 ng of calcium ionophore A23187 per ml for 16 hr (40-kDa subunit) or with 3 μg of human genomic DNA (35-kDa subunit). PCR cycling parameters were as follows. Initial denaturation was at 95°C for 7 min. Low-stringency annealing was performed by cooling to 37°C over 2 min, incubating 2 min at 37°C, heating to 72°C over 2.5 min, extending at 72°C for 1.5 min, heating to 95°C over 1 min, and denaturing at 95°C for 1 min. This cycle was repeated once. Thirty standard cycles (40-kDa subunit) or 40 standard cycles (35-kDa subunit) were performed as follows: 95°C for 1 min, 55°C for 2 min, and 72°C for 2 min. Final extension was at 72°C for 10 min. "Amplicons" of the expected size were gel-purified, subcloned, and sequenced. The 40-kDa subunit cDNAs were isolated by hybridizing the 54-mer amplicon in 5× SSC (1× SSC = 0.15 M NaCl/0.015 M sodium citrate, pH 7) containing 20% formamide at 37°C overnight. Filters were washed in 2× SSC at 42°C for 30 min and exposed to x-ray film. The 35-kDa subunit cDNAs were isolated by hybridizing the 51-mer amplicon in 5× SSC/20% formamide at 37°C overnight. The filters were washed in 2× SSC at 40°C for 30 min and exposed to x-ray film. Positive clones were plaque-purified, their inserts were subcloned into the pBluescript plasmid, and their sequences were determined by using Sequenase.

Expression. cDNAs were separately engineered for expression in vectors containing the simian virus 40 early promoter essentially as described (9). COS cells were transfected with both CLMF subunit expression plasmids mixed together or

Abbreviations: CLMF, cytotoxic lymphocyte maturation factor; rCLMF and nCLMF, recombinant and natural CLMFs; CTL, cytolytic T lymphocyte; IL, interleukin; LAK, lymphokine-activated killer; PHA, phytohemagglutinin; PMA, phorbol 12-myristate 13-acetate; n, natural; PCR, polymerase chain reaction.

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The cDNA sequences reported in this paper have been deposited in the Genbank data base [accession nos. M38443 (35-kDa CLMF subunit) and M38444 (40-kDa CLMF subunit)].

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1 MCPARSLLL^V ATLVL^LDHLS LARNLPVATP DPGMFPC^LH SQNLLRAVSN
 51 MLQKARQTLE FYPCTSEEID HEDITKD^KT^S TVEACLPLEL TKNESCLNSR
 101 ETSFITNGSC LASRKTSFMM ALCLSSIYED LKMYQVEFKT MNAKLLMDPK
 151 RQIFLDQNML AVIDELMQAL NFNSETVPQK SSLEEPDFYK TKIKLCILLH
 201 AFRIRAVTID RVTSYLN^AS

----> <----

1 MCHQQLVISW FSLVFLASPL VAIWELKKDV YVVELDWYPD APGEMVV^LC
 51 DTPEEDGITW TLDQSSEV^LG SGKTLTIQVK EFGDAGQYTC HKGGEVLSHS
 101 LLLLHKKEDG IWSTDILKDQ KEPKNKTFLR CEA^KNYSGRF TC^LWWLTTIST
 151 DLTFSVKSSR GSSDPQGVTC GAATLSAERV RGDNKEYEYS VECQEDSACP
 201 AAEESLPIEV MVDAVHKLKY ENYTSSFFIR DIIKPDP^KPN LQLKPLKNSR
 251 QVEVSWEYPD TWSTPHSYFS LTFCVQVQGK SKREKKDRVF TDKTSATVIC
 301 RKNASISVRA QDRYYSSWS EWASVPC^S

FIG. 1. Amino acid sequences of the 35-kDa (*Upper*) and 40-kDa (*Lower*) CLMF subunits as deduced from the respective cDNAs and shown in single-letter code. Signal peptides are overlined, cysteine residues are marked by a caret, and N-linked glycosylation sites (NXS, NXT, where X is another amino acid) are underlined. The peptide sequences used to generate PCR probes are overlined with arrows indicating the direction of amplification.

with each one separately by the DEAE-dextran method. Twenty-four hours after transfection, the serum-containing medium was replaced with medium containing 1% Nutridoma-SP (Boehringer Mannheim); and supernatant fluids were collected from the cultures after 40 hr. These fluids were stored at 4°C until tested in the bioassays.

General Methods. Standard molecular biological procedures were used as described (10). CLMF bioassays were performed as detailed (7).

Computer Searches. The National Biomedical Research Foundation protein data base (Release 26.0) as well as the Genbank and European Molecular Biology Laboratory databases (Releases 65.0 and 24.0, respectively) were searched for sequences homologous to CLMF cDNAs. The two subunit sequences were compared to each other using the ALIGN program (mutation data matrix, break penalty of 6; see ref. 11).

RESULTS

Partial N-terminal amino acid sequences of the two CLMF subunits (7) were used to generate completely defined 51- to 54-base-pair (bp)-long oligonucleotide probes by means of mixed primer PCR. These probes were used to screen cDNA libraries made from RNA from activated NC-37.98 cells, and cDNAs encoding the two subunits were isolated and characterized. Both cDNAs encode secreted proteins with a classical hydrophobic N-terminal signal peptide immediately followed by the N terminus of the mature protein as determined by protein sequencing (7). Two independent cDNA clones for the 40-kDa subunit were shown to be identical. Both encode the mature 40-kDa subunit that is composed of 306 amino acids (calculated $M_r = 34,699$) and contains 10 cysteine residues and four potential N-linked glycosylation sites (Fig. 1). Two of these sites are within isolated tryptic peptides derived from the purified 40-kDa CLMF subunit protein. Amino acid sequence analysis has shown that Asn-

222 is glycosylated, whereas Asn-125 is not (Fig. 1; F. Podlaski, personal communication). The mature 35-kDa subunit is composed of 197 amino acids (calculated $M_r = 22,513$), with 7 cysteine residues and three potential N-linked glycosylation sites (Fig. 1). When purified CLMF is reduced with 2-mercaptoethanol and analyzed by SDS/PAGE, the 35-kDa subunit appears to be heterogeneous, suggesting that it may be heavily glycosylated (7). Two variants of 35-kDa subunit-encoding cDNAs were isolated. The first type had the sequence shown in Fig. 1. Additional isolates contained what is probably an allelic variation, replacing Thr-213 with a methionine residue.

Computer searches of sequence databases showed that the amino acid sequences of the two subunits are not related to any known protein. The subunit sequences are also not related to each other, since a comparison using the ALIGN program (11) gave a score of 1.27; only scores >3 are considered to indicate significant evolutionary relationship (12). The genes encoding the subunits appear to be unique, since low- and high-stringency hybridizations of genomic blots revealed identical banding patterns (data not shown). RNA blots showed the size of the 40-kDa subunit mRNA to be 2.4 kb, whereas the 35-kDa subunit was encoded by a 1.4-kb transcript (Fig. 2). Expression of the two mRNAs encoding the subunits was coordinately regulated upon induction (Fig. 2). When NC-37.98 cells were activated with PMA and calcium ionophore for 72 hr, mRNA encoding each of the CLMF subunits was minimally detectable at 6 hr after the beginning of induction but was readily detected at 24 hr and continued to accumulate until maximal levels were reached at 72 hr (normalized to GAPDH mRNA levels; see the legend to Fig. 2). In contrast, the mRNA for IL-2 in activated NC-37.98 cells was already at high levels at 6 hr and subsequently decreased, whereas the mRNAs for the low-affinity IL-2 receptor (p55) followed the induction pattern seen for the CLMF subunits. Scanning of RNA blots also revealed that steady-state mRNA levels for the 40-kDa

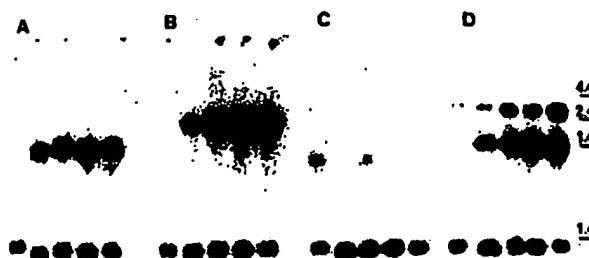


FIG. 2. RNA blots showing the coordinate induction of the 35-kDa (*A*) and 40-kDa (*B*) CLMF subunit mRNAs and IL-2 mRNA (*C*) and its p55 receptor mRNA (*D*). Poly(A)⁺ RNA (5 µg) from NC-37.98 cells activated with 10 ng of PMA and 25 ng of calcium ionophore A23187 per ml were loaded in each lane. Lanes from left to right in each panel show RNAs isolated 6, 24, 30, 48, and 72 hr after induction, respectively. (*Upper*) Four-day exposures. (*Lower*) Two-hour exposure of the same blots after stripping and rehybridization with a GAPDH probe. Marker sizes are in kb (BRL RNA ladder).

CLMF subunit were severalfold higher than those for the 35-kDa subunit expressed by the same cells. This finding parallels the observation that activated NC-37 cells secrete excess free 40-kDa subunit protein (7). The 3' untranslated sequences of both CLMF subunit mRNAs contain several copies of the octamer motif TTATTTAT (data not shown). This sequence is present in other transiently expressed mRNAs and is involved in regulating mRNA stability (13).

Coexpression of the 40-kDa and 35-kDa CLMF subunit cDNAs in COS cells was required to generate secreted biologically active CLMF (Table 1 and Fig. 3). COS cells transfected with cDNA encoding either the 40-kDa subunit alone or the 35-kDa subunit alone did not secrete biologically active CLMF (Table 1). Mixing media conditioned by COS

cells that had been separately transfected with one or the other of the two CLMF subunit cDNAs also did not give rise to bioactive CLMF (Table 1).

Two types of assays were used to compare rCLMF and nCLMF. The first assay measures the proliferation of phytohemagglutinin (PHA)-activated human peripheral blood lymphocytes, whereas the second assay evaluates the synergy between CLMF and suboptimal concentrations of IL-2 in the generation of LAK cells in hydrocortisone-containing cultures (7). The data in Fig. 3 show that rCLMF as expressed in COS cells and nCLMF as purified from NC-37 cells are essentially identical. Dose-response curves for rCLMF and nCLMF were superimposable in each of the two assays, and rCLMF was neutralized by a monoclonal antibody raised against nCLMF. Conditioned media from cultures of mock-transfected COS cells displayed no activity in these assays (Table 1 and data not shown).

DISCUSSION

In a previous report (7), we described the purification of a heterodimeric cytokine, CLMF, that synergized with low amounts of IL-2 to cause the generation of LAK cells in the presence of hydrocortisone and stimulated the proliferation of activated T cells independent of IL-2. In the present report, we have used the N-terminal amino acid sequence information previously obtained to clone the two subunit cDNAs of CLMF. Protein purification of NC-37 cell line-derived CLMF had shown that the protein was composed of two disulfide-bonded subunits with different N-terminal amino acid sequences (7). However, it was not clear from our previous results whether the two subunits were processed from one common gene product and whether proteolytic posttranslational processing other than signal peptide cleavage was occurring. The molecular cloning and sequencing of

Table 1. Coexpression of both CLMF subunit cDNAs is required for secretion of biologically active CLMF by COS cells

Addition	Conc., units/ml	Dilution	[³ H]Thymidine incorporated by PHA-activated lymphoblasts, mean cpm ± 1 SEM
Cytokine*			
None	—		11,744 ± 514
nCLMF	200		68,848 ± 878
nCLMF	40		48,827 ± 605
nCLMF	8		26,828 ± 594
nCLMF	1.6		17,941 ± 196
Culture fluid from COS cells transfected with			
A. 35-kDa CLMF subunit cDNA		1:20	11,912 ± 660
		1:100	10,876 ± 232
B. 40-kDa CLMF subunit cDNA		1:20	11,699 ± 931
		1:100	11,666 ± 469
C. 35-kDa + 40-kDa CLMF subunit cDNAs		1:20	58,615 ± 587
		1:100	38,361 ± 828
1:1 mix of culture fluids A and B		1:10 [†]	11,544 ± 483
		1:50	10,503 ± 259
CM from mock-transfected control‡		1:20	11,503 ± 286
		1:100	10,751 ± 303

PHA-activated lymphoblasts were prepared from human peripheral blood mononuclear cells as described (7). Lymphoblast proliferation was measured in a 48-hr assay (7) in which 2×10^4 lymphoblasts were incubated in 100-µl cultures containing the indicated amounts of natural CLMF (nCLMF) or COS cell culture fluids. [³H]Thymidine was added to each culture 18 hr prior to harvest. Conc., concentration.

*nCLMF is purified NC-37-derived CLMF.

[†]1:10 dilution of the 1:1 mixture of culture fluids A and B was equivalent to a 1:20 final dilution of each of the individual culture fluids.

[‡]Conditioned medium (CM) from cultures of mock transfected COS cells.

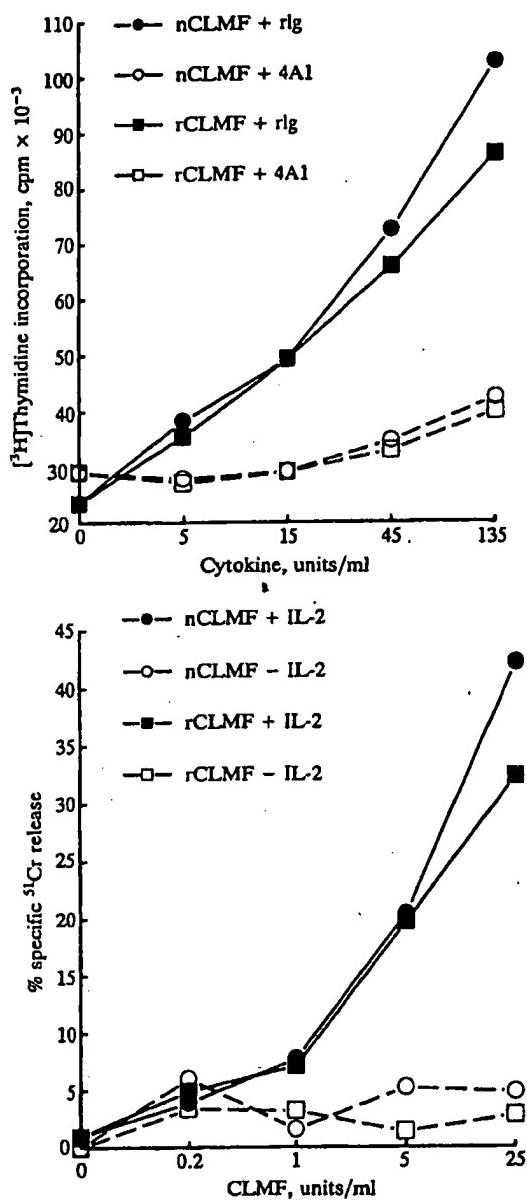


Fig. 3. Comparison of biologic activities of nCLMF (circles) and recombinant CLMF (rCLMF, squares). nCLMF was purified from NC-37 cell-conditioned media; rCLMF was produced from conditioned media from cultures of COS cells transfected with cDNAs encoding the 40-kDa and 35-kDa human CLMF subunits. (*Upper*) T-cell growth factor assay. The ability of CLMF to stimulate the proliferation of human PHA-activated lymphoblasts in 48-hr cultures was assayed as described (7). CLMF preparations were mixed with neutralizing rat monoclonal anti-human CLMF antibody 4A1 (ref. 7; open symbols) or with normal rat IgG (Sigma; IgG, closed symbols) at a final concentration of 20 µg of IgG/ml and were incubated for 30 min at 37°C prior to addition of PHA blasts. All values are means of triplicate determinations. (*Lower*) LAK cell induction assay. The ability of CLMF, alone or in combination with recombinant IL-2, to induce the generation of LAK cells in 4-day cultures was assessed as described (7). Low-density peripheral blood lymphocytes were incubated in the presence of various amounts of nCLMF or rCLMF with (closed symbols) or without (open symbols) recombinant IL-2 at 7.5 units/ml. Units of CLMF activity were based on previous titrations in the T-cell growth factor assay. Hydrocortisone sodium succinate (Sigma) was included at a concentration of 0.1 mM to minimize triggering of endogenous cytokine cascades. Lysis of ⁵¹Cr-labeled Daudi cells was assessed at an effector/target ratio of 5:1. The data shown represent the means of quadruplicate determinations. The spontaneous ⁵¹Cr release was 20%.

the corresponding cDNAs now has demonstrated that there is no common precursor for the two CLMF subunits; rather, they are encoded by completely different genes. The predicted and actual amino acid composition for the two subunits are strikingly similar; differences in predicted versus actual molecular weights are accounted for by glycosylation (F. Podlaski, personal communication). Thus, the only major posttranslational proteolytic event that appears to take place in the maturation of the CLMF subunits is signal peptide cleavage.

The kinetics of expression of the individual CLMF subunit mRNAs were examined and compared to the expression of mRNAs for IL-2 and the IL-2 receptor p55. Previously it had been observed that NC-37 cells, like certain murine (14) and marmoset (15) B-cell lines, secreted IL-2 when activated (M.K.G., unpublished results). RNA blots demonstrated that upon activation of NC-37 cells, both CLMF subunit mRNAs were coordinately induced with kinetics similar to the IL-2 receptor (p55) mRNAs. On the other hand, IL-2 mRNA levels peaked much earlier. Similar differences in induction kinetics were also seen at the level of IL-2 and CLMF bioactivity secreted from NC-37 cells (M.K.G., unpublished data). These kinetic differences are consistent with our previous observation that in a cytolytic lymphocyte response, IL-2 appears to act earlier than CLMF (5).

Transfection studies with COS cells established that only coexpression of both subunit cDNAs gives rise to secreted bioactive CLMF. Thus, it appears that the two proteins have to interact within the endoplasmic reticulum to assemble properly into bioactive secreted CLMF. By comparing the activity of rCLMF to that of nCLMF in the T-cell growth factor and LAK cell induction assays (Fig. 3) and assuming that the specific activity of rCLMF is similar to that of nCLMF [8×10^7 units/mg (7)], we estimate that the amount of rCLMF heterodimer produced in these experiments was 5–50 ng/ml. The finding that COS cells, which are fibroblast-like cells, are able to assemble correctly the two CLMF subunits to form bioactive CLMF indicates that this secretion and processing pattern is not limited to cells of the lymphoid lineage.

Western blot analysis using an anti-CLMF antibody specific for the 40-kDa subunit has allowed confirmation that (i) COS cells transfected with both CLMF subunit cDNAs secrete CLMF with the expected heterodimeric structure and (ii) COS cells transfected with the 40-kDa subunit cDNA alone secrete that subunit (F. Podlaski, personal communication). Since no bioactivity was detected in media conditioned by COS cells transfected with only the 40-kDa subunit, that subunit by itself appears either to have a much reduced specific activity compared with heterodimeric CLMF or to be completely inactive.

Because of the lack of a high-affinity antibody specific for the 35-kDa subunit, we have not yet been able to determine definitively whether COS cells transfected with only the 35-kDa subunit cDNA secrete that subunit. Since no bioactivity was detected in the media, secretion of a bioactive 35-kDa subunit by itself could be very inefficient; alternatively, similar to the 40-kDa subunit, the protein could be much less active or inactive altogether. Intracellular 35-kDa protein in the absence of the other subunit could be inherently unstable; there is precedence for this phenomenon, since it has been reported that 90% of the β chains of lutropin (LH), when expressed in the absence of α chains, are retained in the endoplasmic reticulum and are slowly degraded (16). Simple mixing of media conditioned by COS cells transfected separately with either one of the two CLMF subunit cDNAs did not yield bioactive CLMF. One possible explanation would be that the cells do not secrete the 35-kDa CLMF subunit by itself. More likely, our experimental conditions did not allow proper heterodimer formation. One would expect that only

carefully controlled renaturation and oxidation conditions would allow the disulfide bond formation required for generation of bioactive CLMF.

Normal human peripheral blood lymphocytes under the appropriate induction conditions produce both CLMF subunit mRNAs and secrete the active protein (N.N. and M.K.G., unpublished data). There is some evidence suggesting that CLMF is produced predominantly by B cells. In preliminary experiments, B-cell mitogens have appeared to be more effective than T-cell mitogens in eliciting CLMF production from peripheral blood lymphocytes (M.K.G., unpublished results). When screening human cell lines for their ability to produce CLMF activity (7), we observed that four of eight B-cell lines tested produced CLMF after activation with PMA and calcium ionophore, whereas none of five T-cell lines produced CLMF. Nevertheless, three of these T-cell lines secreted large amounts of IL-2 and tumor necrosis factor activity after activation (M.K.G., unpublished results). Likewise, natural killer cell stimulatory factor (NKSF), a heterodimeric cytokine similar or identical to CLMF, was isolated from RPMI 8866 lymphoblastoid B cells (17). A recent report (18) has indicated that B lymphocytes can secrete a cytokine(s) distinct from IL-2 that facilitates virus-specific cytolytic T-lymphocyte responses. It is possible that CLMF may have been the cytokine active in those studies. Thus, although B lymphocytes have not traditionally been viewed as cytokine-producing helper cells, it is conceivable that CLMF production constitutes a novel mechanism whereby B lymphocytes contribute to the amplification of T-lymphocyte responses. In addition to the biologic activities described in this report, CLMF by itself has been shown (i) to activate NK cells in an 18–22 hr assay, (ii) to facilitate the generation of specific allogeneic CTL responses, and (iii) to stimulate the secretion of γ interferon by resting peripheral blood lymphocytes (M.K.G., unpublished results). It can also synergize with low concentrations of recombinant IL-2 in the latter two assays and in causing the proliferation of resting peripheral blood lymphocytes. In view of its production by peripheral blood lymphocytes and its diverse actions on lymphoid cells, it appears that CLMF constitutes a new interleukin. We propose that CLMF be

given the provisional designation IL-12. The availability of recombinant CLMF will now make possible a broader and more detailed characterization of its biology.

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Immunization With Melan-A Peptide-Pulsed Peripheral Blood Mononuclear Cells Plus Recombinant Human Interleukin-12 Induces Clinical Activity and T-Cell Responses in Advanced Melanoma

By Amy C. Peterson, Helena Harlin, and Thomas F. Gajewski

Purpose: Preclinical studies showed that immunization with peripheral blood mononuclear cells (PBMC) loaded with tumor antigen peptides plus interleukin-12 (IL-12) induced CD8⁺ T-cell responses and tumor rejection. We recently determined that recombinant human (rh) IL-12 at 30 to 100 ng/kg is effective as a vaccine adjuvant in patients. A phase II study of immunization with Melan-A peptide-pulsed PBMC + rhIL-12 was conducted in 20 patients with advanced melanoma.

Patients and Methods: Patients were HLA-A2-positive and had documented Melan-A expression. Immunization was performed every 3 weeks with clinical re-evaluation every three cycles. Immune responses were measured by ELISpot assay before and after treatment and through the first three cycles, and were correlated with clinical outcome.

Results: Most patients had received prior therapy and had visceral metastases. Nonetheless, two patients achieved a

complete response, five patients achieved a minor or mixed response, and four patients had stable disease. The median survival was 12.25 months for all patients and was not yet reached for those with a normal lactate dehydrogenase. There were no grade 3 or 4 toxicities. Measurement of specific CD8⁺ T-cell responses by direct ex vivo ELISpot revealed a significant increase in interferon gamma-producing T cells against Melan-A ($P = .015$) after vaccination, but not against an Epstein-Barr virus control peptide ($P = .86$). There was a correlation between the magnitude of the increase in Melan-A-specific cells and clinical response ($P = .046$).

Conclusion: This immunization approach may be more straightforward than dendritic cell strategies and seems to have clinical activity that can be correlated to a biologic end point.

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MOST MELANOMA tumors express antigens that can be recognized by CD8⁺ T cells.^{1,2} Nonetheless, tumors frequently escape immune destruction, either from a failure to generate an optimal tumor antigen-specific T-cell response or from development of resistance to the T-cell response induced. One strategy to overcome the former hurdle is through active immunization, the opportunity for which has been facilitated by the molecular definition of melanoma antigens.³ Specific CD8⁺ T cells that are properly activated can home to tumor sites and kill tumor cells, to the extent to which they can overcome negative immunoregulatory pathways and tumor resistance.⁴

The optimal immunization strategy for inducing tumor antigen-specific CD8⁺ effector T cells in humans remains undefined. However, antigen-presenting cell-based strategies have shown promise. Both monocyte-derived^{5,6} and bone marrow-derived⁷ dendritic cells (DCs) have been loaded with

melanoma tumor antigens and administered in the advanced-disease setting, with evidence for immunization and tumor regression in subsets of patients. However, DCs are cumbersome to generate and alternative approaches that are more straightforward yet equally as effective would be useful. One cofactor produced by DCs that contributes to their efficacy is interleukin-12 (IL-12), which facilitates the induction of interferon gamma (IFN- γ)-producing cytolytic effector cells.⁸⁻¹⁰ Endogenous IL-12 seems necessary for optimal rejection of immunogenic murine tumors^{11,12} and provision of exogenous IL-12, either alone¹³ or combined with tumor antigen-based vaccines,¹⁴⁻¹⁹ can induce rejection of pre-established tumors in murine models. We previously have shown that coadministration of IL-12 with peripheral blood mononuclear cells (PBMCs) loaded with tumor antigen peptides induced specific cytolytic T-lymphocyte responses and tumor protection in mice, circumventing the need to generate dendritic cells.²⁰ The ease by which PBMC can be isolated from patients has made this an attractive approach for clinical translation. We recently conducted a phase I clinical study to determine the dose of recombinant human (rh) IL-12 necessary to induce T-cell responses in combination with antigen-loaded PBMCs, and found that doses from 30 to 100 ng/kg administered subcutaneously (sc) at the vaccine site were optimal and well tolerated.²¹ The effective range of doses indicated that a straight dose of 4 μ g might be used.

In this article, we describe results of a phase II clinical study of immunization with Melan-A/MART-1³ peptide-pulsed autologous PBMCs + rhIL-12 in HLA-A2-positive patients with

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advanced melanoma. Immune responses were analyzed using a direct ex vivo ELISpot assay. We show that this vaccine approach had clinical activity and that the magnitude of increased T-cell response correlated with clinical outcome.

PATIENTS AND METHODS

Patient Enrollment and Eligibility

This was an open-label, nonrandomized, single-institution study of Melan-A peptide-pulsed autologous PBMCs + rhIL-12.⁴ The protocol was approved by the University of Chicago Institutional Review Board and all patients signed written informed consent. Patients who were both HLA-A2-positive and showed Melan-A tumor expression by reverse transcriptase polymerase chain reaction (RT-PCR) were considered for inclusion. Additional inclusion criteria were life expectancy more than 12 weeks, Karnofsky performance status ≥ 70 , and adequate hematopoietic, renal, and hepatic function. Delayed-type hypersensitivity (DTH) skin testing was performed against mumps, *Candida*, and *Trichophyton*, not for eligibility but to correlate subsequently with clinical outcome and immunization potential. Patients were excluded if they had severe cardiovascular disease or arrhythmia, were pregnant or nursing, had biologic therapy received within 4 weeks, tested positive for hepatitis B surface antigen or human immunodeficiency virus (HIV), had clinically significant autoimmune disease or any illness requiring immunosuppressive therapy, had a psychiatric illness that would interfere with patient compliance and informed consent, had active gastrointestinal bleeding or uncontrolled peptic ulcer disease, or had uncontrolled brain metastases. Patients with treated brain metastases who were clinically and radiographically stable and did not require corticosteroids were allowed to enter onto the trial.

Patient Characteristics

Twenty patients with metastatic melanoma were enrolled after giving written informed consent. Patient characteristics are outlined in Table 1. All patients had advanced disease; the majority had at least three sites of metastasis, 60% of which were visceral (ie, noncutaneous and nonpulmonary metastases). Approximately two thirds of the patients had received prior therapy, and 10 patients had an elevated lactate dehydrogenase (LDH) level, which is an important negative prognostic factor.²² Only 45% were positive for at least one recall antigen (mumps, *Candida*, or *Trichophyton*) by DTH skin testing.

RT-PCR Analysis

mRNA was isolated from fresh tumor cells using guanidine and cesium chloride. cDNA was synthesized and PCR was performed for Melan-A and beta-actin using the primer pairs and reaction conditions described previously.²¹ Control reactions without reverse transcriptase were performed to rule out a contribution of genomic DNA. PCR products were visualized using a 1.5% ethidium bromide-stained agarose gel. No formal quantitation was performed.

Vaccine Preparation

Therapy consisted initially of three 21-day cycles. Vaccinations were given on the first day of each cycle and rhIL-12 was administered subcutaneously on days 1, 3, and 5. Approximately 100 to 150 mL of peripheral blood from patients was collected on day 1 of each cycle into heparinized 60-mL syringes using sterile technique. PBMCs were isolated over a Lymphoprep gradient (Lymphoprep; Axis-Shield PoC, Oslo, Norway), counted, washed, and resuspended in Dulbecco's phosphate-buffered saline (DPBS) at 40×10^6 cells/mL. At least 10×10^6 cells from each sample were cryopreserved to prepare CD8⁺ and CD8⁻ fractions for subsequent correlative immunologic studies. The Melan-A₂₇₋₃₅ peptide (AAGIGILTV) was produced according to good manufacturing practice standards by Multiple Peptide Systems (San Diego, CA) and provided in lyophilized vials. Aliquots of peptide were prepared at 5 mmol/L in dimethyl sulfoxide and stored at

Table 1. Patient Characteristics

Patient Characteristic	Patients (n = 20)	
	No.	%
Age, years		
Median	58	
Range	35-79	
Sex		
Male	9	45
Female	11	55
Karnofsky performance status (ECOG)		
90%-100% (0)	10	50
70%-80% (1)	9	45
60%-70% (2)	1	5
No. of metastatic sites		
1	2	10
2		None
≥ 3	18	90
Location of metastases		
Visceral	13	65
Brain (treated)	4	20
Prior therapy		
None	6	30
Chemotherapy or immunotherapy	7	35
As only prior therapy	5	25
Chemotherapy	1	5
As only prior therapy	1	5
Immunotherapy	4	20
As only prior therapy	1	5
Other*	2	10
As only prior therapy		None
Adjuvant IFN- α	5	25
As only prior therapy	3	15
Elevated LDH	10	50
DTH recall positive	9	45

Abbreviations: ECOG, Eastern Cooperative Oncology Group; IFN- α , interferon alfa-2b; LDH, lactate dehydrogenase; DTH, delayed-type hypersensitivity.

*Experimental therapy other than a melanoma vaccine, immunomodulatory cytokine, or chemotherapy.

-80°C for up to 3 months. Peptide preparations were quality controlled for HLA-A2 binding, sterility, and identity by high-performance liquid chromatography and mass spectrometry. An aliquot of peptide was diluted to 20 $\mu\text{mol/L}$ in DPBS and mixed with an equal volume of patient PBMCs (final peptide concentration, 10 $\mu\text{mol/L}$; target number of PBMCs, 10^8) followed by incubation at 37°C for 1 hour in 10 mL DPBS. The cells were then irradiated (20 Gy), washed in DPBS, and resuspended in 1 mL DPBS. The suspension of peptide-loaded PBMCs was injected sc using a 1-mL syringe and a 21-gauge needle, divided evenly into two sites. Preferred sites were those near draining lymph node basins but not near a tumor mass. The actual number of PBMCs administered per vaccine ranged from 78 to 100×10^6 .

rhIL-12 was provided by Genetics Institute (Cambridge, MA) as a lyophilized powder of 10 μg under vacuum. Each vial was intended for single use only and was stored as a powder in our research pharmacy at 2 to 8°C until reconstituted with sterile water for injection. Once reconstituted, rhIL-12 was loaded into 3-mL syringes and used within 4 hours. rhIL-12 (4 μg) was administered sc with a 25-gauge needle just after pulsed PBMC inoculation and immediately adjacent to one of the two immunization sites on days 1, 3, and 5. The same approximate location was used for each injection of peptide-pulsed PBMCs and rhIL-12 for each cycle.

Toxicity Assessment and Criteria for Clinical Response

Toxicities were determined using the National Cancer Institute common toxicity criteria scale version 2.0. A complete response (CR) was assigned if there was disappearance of all lesions without the appearance of any new

lesions; a partial response (PR) was defined as $\geq 50\%$ reduction in total tumor volume; a minor response (MR) was defined as less than 50% reduction in total tumor volume; progressive disease (PD) was assigned if new lesions appeared, any tumor reappeared, or if a 25% increase in tumor area was observed; a mixed response was assigned if at least one tumor decreased in size with other or new tumors growing; stable disease (SD) was anything that did not fit the aforementioned criteria. When possible, cutaneous lesions were photographed.

CD8⁺ T-Cell Preparation

CD8⁺ and CD8⁻ fractions from PBMC were isolated at the time of preparation of each vaccine and cryopreserved until analysis in batch fashion. CD8⁺ T lymphocytes were isolated by positive selection using CD8 microbeads and magnetic columns (MACS system; Miltenyi Biotech, Auburn, CA). The unbound CD8⁻ fraction was cryopreserved for use as antigen-presenting cells for in vitro expansion of specific CD8⁺ T cells. Although the primary ELISpot analysis was performed directly with thawed cells, a secondary assay was carried out after in vitro expansion. For in vitro expansion, CD8⁻ cells were thawed from each time point and pooled, pulsed with 50 $\mu\text{mol/L}$ Melan-A peptide in serum-free Iscove's modified Dulbecco's medium (IMDM) with beta₂-microglobulin, irradiated (3,000 rad), washed, and plated at 2×10^6 cells/well in 24-well plates. CD8⁺ T cells were thawed and cultured with the irradiated CD8⁻ cells at 4×10^5 cells/well in IMDM medium containing 10% human AB serum. After 5 days, the cells were collected and plated with a new batch of Melan-A-pulsed irradiated CD8⁻ cells. After an additional 5 days the cells were collected and tested.

ELISpot Assays

Briefly, 96-well membrane bottomed plates (MAHA S4510; Millipore, Bedford, MA) were coated with 15 $\mu\text{g/mL}$ of antihuman IFN- γ antibody (Mabtech, Cincinnati, OH) in PBS. The plates were washed and CD8⁺ T cells, either freshly thawed at 5×10^4 cells/well or after in vitro expansion at 5×10^3 cells/well, were plated in triplicate in IMDM medium with 10% human AB serum. T2 cells (transporter associated with antigen processing-deficient cell line, American Type Culture Collection no. CRI 1992) were pulsed for 1 hour at 37°C with 50 $\mu\text{mol/L}$ peptide (either derived from HIV [ILKEPVHGV], Epstein-Barr virus [EBV; GLCTLVAML], or Melan-A [AAGIGILTV]), washed, and plated at a 5-to-1 ratio to the T cells. A replicate of CD8⁺ T cells was stimulated with PMA (phorbol 12-myristate 13-acetate) (50 ng/mL) + ionomycin (0.5 $\mu\text{g/mL}$) as a positive control. After 24 hours, the cells were removed by washing with PBS + 0.05% Tween (wash buffer), and biotinylated antihuman IFN- γ antibody was added in PBS + 0.5% fetal calf serum. The plates were incubated for 2 to 4 hours at room temperature, washed, and streptavidin-alkaline phosphatase was added for 1 hour at room temperature. The plates were then washed, BCIP-NBT (5-bromo-4-chloro-3-indolyl phosphate/nitro-blue tetrazolium) was added, and the plates were finally washed with water and allowed to air dry. Plates were scanned with an ELISpot reader (CTL Technologies, Cleveland, OH) and the number of spots per well was enumerated after the background was set on the basis of wells that had been incubated with medium alone; spot separation was adjusted using Immunospot software (CTL Technologies). For each sample, the number of T cells producing IFN- γ in response to EBV or Melan-A peptides was determined by subtracting the number of spots seen in response to HIV peptide. The mean and SD were determined for each triplicate sample. After immunization, the time point at which peak frequencies among the first three cycles were observed was used for data analysis.

Statistical Analysis

Comparisons between pre- and post-ELISpot frequencies were performed using a paired *t* test, and comparisons of augmented ELISpot frequencies between responders and nonresponders were made using an unpaired two-sided *t* test. Correlations between various dichotomous variables and clinical outcome were made using Fisher's exact test (two-sided). Survival data were determined using the Kaplan-Meier method, with differences among subgroups assessed by the log-rank test. All analyses were performed using SPSS software (version 8.0; SPSS Inc, Chicago, IL).

Table 2. Adverse Events

Adverse Event	Grade 1	Grade 2	Grade 3
Fatigue	16	0	0
Anorexia	6	0	0
Fever	7	0	0
Rash	3	0	0
Headache	3	0	0
Nausea	2	0	0
Injection site reaction	5	0	0
Neutropenia	1	2	0
Thrombocytopenia	2	0	0
Hepatic	5	2	0
Creatinine	1	0	0

NOTE. Adverse events were determined using the National Cancer Institute common toxicity criteria scale version 2.0.

RESULTS

Immunization Treatment and Toxicities

Each 3-week cycle consisted of immunization on day 1 and sc rhIL-12 administration on days 1, 3, and 5, as described in Methods. Three cycles constituted one course of therapy and patients were evaluated for response after each course. Patients were observed as inpatients in our General Clinical Research Center for the first 24 hours of each cycle.

Adverse reactions are listed in Table 2. All but one patient completed at least three cycles of therapy. There were no grade 3 to 4 toxicities; two patients had grade 2 neutropenia and two patients had grade 2 ALT or AST elevations, which were reversible. The most common adverse reactions were fatigue and fever.

Clinical Outcome

Clinical response outcomes are listed in Table 3. Two patients had a CR, for an overall response rate of 10%. In addition, four patients (20%) had a mixed response, one patient (5%) had an MR, four patients (20%) had SD, and the remaining nine patients (45%) had PD. The sites of tumor response were diverse. The two patients who experienced a CR both had numerous metastases of 2 cm or less and a normal LDH. One patient was female, had multiple cutaneous lesions, and no prior therapy; the other patient was male, had multiple lung lesions, and had experienced prior treatment failure from chemoimmunotherapy. Neither patient experienced a recurrence with a mean follow-up time of 28 months at the time of data analysis. Of the five other patients who showed a decrease in size of at least one tumor mass, three had responses in skin, one had a response in bone, and one had a response in an adrenal lesion. Three of the four patients with SD had visceral metastases.

Table 3. Clinical Outcome

Best Response	No. of Patients	%
Complete response	2	10
Partial response	0	0
Minor response	1	5
Mixed response	4	20
Stable disease	4	20
Progressive disease	9	45

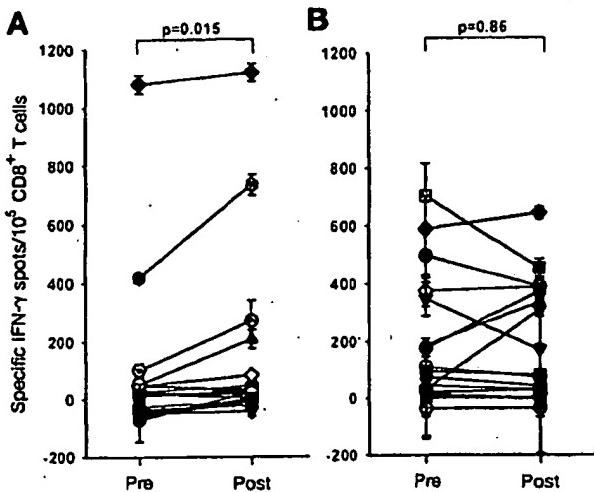


Fig 1. Interferon gamma ELISpot frequencies by $CD8^+$ T cells against Melan-A and (A) Epstein-Barr virus (EBV) (B) pre- and postimmunization. Control values with HIV peptide were subtracted out. Post- and pretreatment values were compared using a paired t test.

Peptide-Specific T-Cell Responses by ELISpot

A carefully controlled IFN- γ ELISpot assay was used to monitor the immune response to immunization. Cryopreserved $CD8^+$ T cells were thawed in batch fashion and stimulated in triplicate directly ex vivo with T2 cells loaded with peptides derived from either HIV, EBV, or Melan-A. The HIV values were subtracted from those obtained with either Melan-A or EBV as an internal control at each time point. Seventeen of the enrolled patients had adequate cryopreserved material with which to perform immunologic assessments.

As shown in Fig 1, some patients displayed a high frequency of Melan-A-specific $CD8^+$ T cells before vaccination, with as high as 1% of $CD8^+$ cells responding to this peptide. These T cells were functional because they produced IFN- γ . The majority of patients showed an increase in the frequency of Melan-A-specific cells after immunization ($P = .015$). In contrast, the frequencies of specific $CD8^+$ T cells responding to the EBV peptide did not vary significantly overall ($P = .86$). Although the changes in T-cell frequency were modest, these results demonstrate an antigen-specific response after immunization with Melan-A peptide-pulsed PBMC + rhIL-12.

The changes in Melan-A-specific ELISpot frequencies were compared among patients who had a mixed response or better and those who had no clinical response. As shown in Fig 2, the mean increase in Melan-A-specific T cells for the clinical responders was 112 ± 45 and for nonresponders was 26 ± 16 , indicating that a greater absolute increase in Melan-A-specific T cells was associated with tumor regression ($P = .046$).

Survival and Associations Between Immunologic Parameters and Clinical Outcome

The overall median survival was 12.25 months and is shown in Fig 3A. Seven patients remained alive at the time of data analysis, with all patients followed beyond 12 months. Because the presence of elevated levels of serum LDH is a known

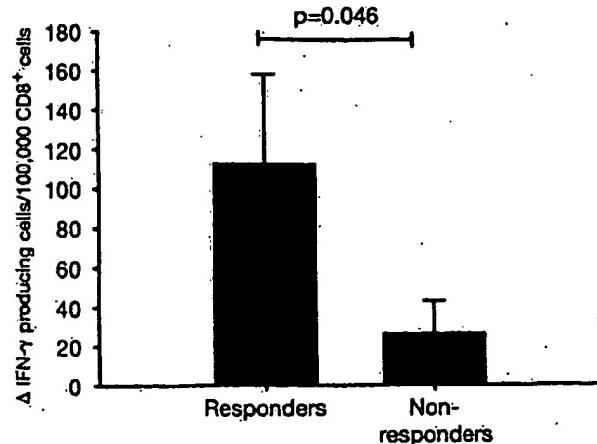


Fig 2. Comparison of increased Melan-A ELISpot frequencies after immunization between clinical responders and nonresponders. The absolute difference between Melan-A-specific ELISpot frequencies post- and pretreatment was compared between responders and nonresponders using a two-sided, unpaired t test.

negative prognostic factor,²³ survival was also compared in response to this vaccine on the basis of LDH level (Fig 3B). The median survival for patients with an elevated LDH level was 9.25 months, whereas the median had not yet been reached for those with a normal LDH ($P = .005$). In addition, the median survival for patients who experienced a significant increase in Melan-A-specific T cells was not yet reached, compared with 8.5 months for patients without a significant increase in Melan-A-specific cells (Fig 3C; $P = .120$).

Additional immunologic parameters that had been measured were also analyzed for associations with either clinical response or survival and are summarized in Table 4. Neither a positive recall DTH to standard antigens nor a relatively high number of EBV- or Melan-A-specific $CD8^+$ T cells before immunization correlated with either outcome. The median pretreatment Melan-A-specific T cell frequency was 23 in clinical nonresponders and -26 in responders. To increase the sensitivity of the assay to detect Melan-A-specific T cells, an in vitro expansion was performed on the preimmunization samples and analyzed by ELISpot as described in Methods. Ten patients showed high Melan-A-specific T cell frequencies after in vitro expansion. However, this also failed to correlate with clinical outcome. Finally, although a normal LDH level was associated with survival, it did not correlate with clinical response and also did not correlate with immune response. Collectively, these results reinforce the specificity of the result showing a significant association between an increased number of Melan-A-specific T cells and clinical outcome.

Expression of Melan-A in Resected Tumors After Immunization

It was conceivable that some patients developed PD despite immunization because of outgrowth of Melan-A-negative tumor cells. Posttreatment tumor samples were obtained from progressing tumors from three patients and analyzed by RT-PCR. Although the new metastasis that developed in patient 1 was negative for

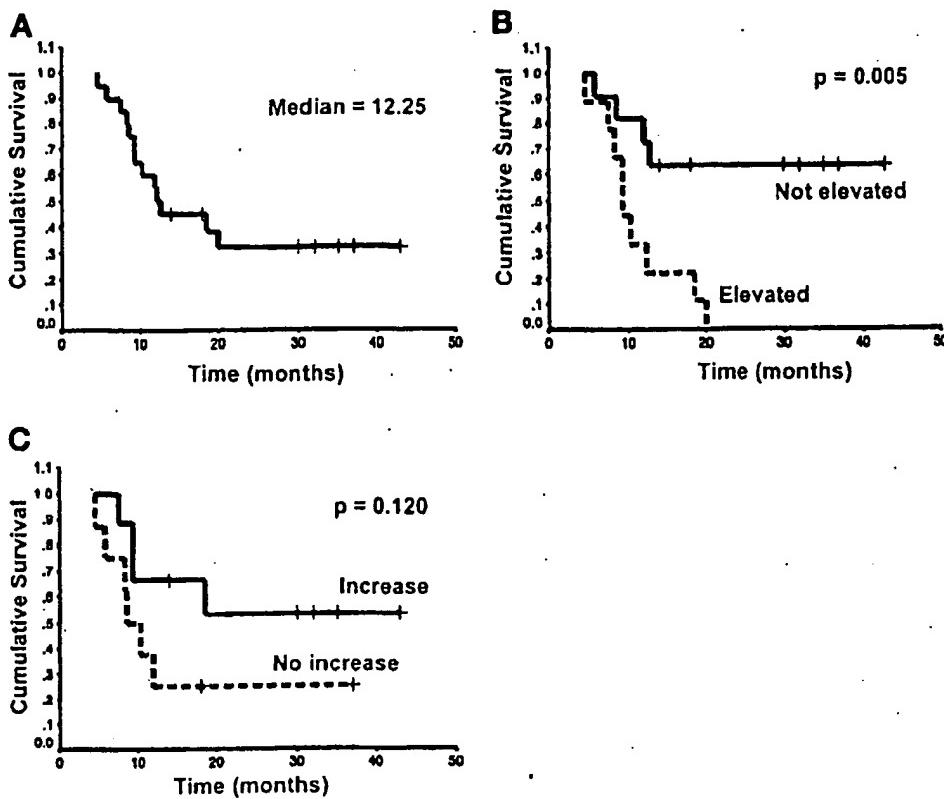


Fig 3. Overall survival for all patients (A), on the basis of serum lactate dehydrogenase greater than 200 U/L (B), and on the basis of increased Melan-A-specific interferon gamma-producing CD8⁺ T cells (C) was determined using the Kaplan-Meier method. Differences between groups were compared using the log-rank test.

Melan-A expression, those samples from patients 4 and 6 retained detectable expression of Melan-A mRNA (Fig 1). These results indicate that, although outgrowth of antigen-negative tumors can occur, other mechanisms of resistance to immune destruction likely explain the lack of clinical response in other patients.

DISCUSSION

In this study we used Melan-A peptide-pulsed autologous PBMC + rhIL-12 as a vaccine to treat HLA-A2-positive patients with advanced melanoma. We observed a significant increase in Melan-A-specific IFN- γ -producing CD8⁺ T cells after immunization, and found a statistical association between clinical response and the magnitude of the specific T-cell increase. Although it is difficult to compare across individual, small phase II studies, these results are similar to those that have been reported using antigen-loaded dendritic cells, but with a strategy that may be more straightforward to execute.

Preparation of the peptide-loaded PBMCs typically took 5 hours from phlebotomy to injection, and quality control of the cell product was facilitated by the lack of an extended in vitro culture period and absence of exposure to culture medium or serum proteins that is required for dendritic cell preparations. Conversely, dendritic cell vaccines have been prepared in batches and cryopreserved in individual doses in some studies, which obviates the need to prepare a fresh vaccine at each time point. Cryopreservation of vaccines has not yet been examined with our current approach. A comparative trial between PBMC/rhIL-12 and dendritic cell-based vaccination may, therefore, be of interest as the technologies continue to develop. Our results

support the notion developed in preclinical models that IL-12 can contribute to effective antitumor immunity, and are consistent with the results of a recent adjuvant vaccine study using rhIL-12 in melanoma.²⁴

We used a direct ex vivo ELISpot assay to assess antigen-specific T-cell responses in this study. Control experiments testing EBV reactivity from normal donors revealed that ELISpot analysis could be performed accurately on cryopreserved CD8⁺ T cell samples immediately after thawing (H. Harlin and T. Gajewski, unpublished data). We found that background reactivity against the control HIV peptide varied among patients and to some extent among time points for an individual patient. The magnitude of increase in apparent Melan-A-reactive T cells would have been greater in some patients had the values obtained with the HIV control peptide not been subtracted. We believe that this experimental detail is critical because it normalizes the samples for background differences and provides an internal control for minor variation between individual vials of cryopreserved T cells. We also compared the Melan-A frequencies to those against an EBV control peptide, to determine whether the treatment was altering ELISpot results. We performed our analyses on purified CD8⁺ T cells to control for variable numbers between patients and across time points. It is possible that we excluded subpopulations of CD8⁻ T cells, CD4⁺ T cells, and natural killer T cells that could have produced IFN- γ in response to Melan-A. Nonetheless, our results revealed a measurable and significant increase in Melan-A-specific T cells posttreatment. Our currently employed ELISpot assay is distinct from the assay used in our phase I trial of peptide-pulsed

Table 4. Statistical Correlates With Response or Survival

Parameter	Correlation With Response (<i>P</i>)	Correlation With Survival (<i>P</i>)
Positive DTH recall	.642	.130
Strong EBV pre-Rx*	.131	.491
Increased EBV post versus pret	.290	.644
Strong Melan-A pre-Rx†	.644	.481
Increased Melan-A post versus pret	.046	.120
Strong in vitro expansion of Melan-A§	.304	.565
LDH levels < 200	.99	.005

NOTE. Associations with response were determined using Fisher's exact test (two-sided), except the differences between pre- and posttreatment, which were determined using an unpaired *t* test. Associations with survival were determined using the Kaplan-Meier method and log-rank test. Significant values are indicated in boldface.

Abbreviations: DTH, delayed-type hypersensitivity; EBV, Epstein-Barr virus; Rx, immunization; LDH, lactate dehydrogenase; HIV, human immunodeficiency virus; IL-2, interleukin-2.

*At least 90 spots per 10^5 CD8 $^+$ T cells after subtraction of background against a control HIV peptide.

†Changes between post- and prevaccination samples were calculated as the difference between the absolute number of specific spots and compared using an unpaired *t* test between clinical responders and nonresponders.

‡At least 40 spots per 10^5 CD8 $^+$ T cells after subtraction of background against a control HIV peptide.

§At least 90 spots per 10^5 CD8 $^+$ T cells after subtraction of background against a control HIV peptide, after a 10-day in vitro expansion with Melan-A peptide-pulsed autologous CD8 $^+$ cells and IL-2.

PBMC + rhIL-12 and in other trials^{21,25} in which in vitro expansion had been performed before assessment of IFN- γ production. Analysis of T-cell responses with minimal in vitro manipulation should most accurately reflect the status of those cells *in vivo*.

High frequencies of Melan-A-specific, IFN- γ -producing CD8 $^+$ T cells were observed in some patients at study entry when they clearly had progressively growing melanoma. This observation indicates that the absolute frequency of functional T cells against a tumor antigen does not correlate with the behavior of the tumor. We also found no statistical association between this high frequency and clinical outcome; in fact, the two patients who experienced a CR had undetectable Melan-A-specific T cells before therapy. Although high frequencies of T cells reacting with a Melan-A tetramer have been detected in some normal donors,²⁶ those cells had a naïve surface phenotype and did not produce high levels of IFN- γ . What did correlate with clinical response in our current study is a meaningful increase in Melan-A-specific T cells posttreatment. These increases were modest (a net gain of 112 spots per 10^5 CD8 $^+$ T cells on average), indicating either that a subtle alteration in the steady-state between the immune response and a growing tumor in favor of increased T-cell frequencies is sufficient to translate into tumor regression, or that another immune function that we are not measuring is contributing to the final event of tumor shrinkage. Tumor regressions without detectable increases in

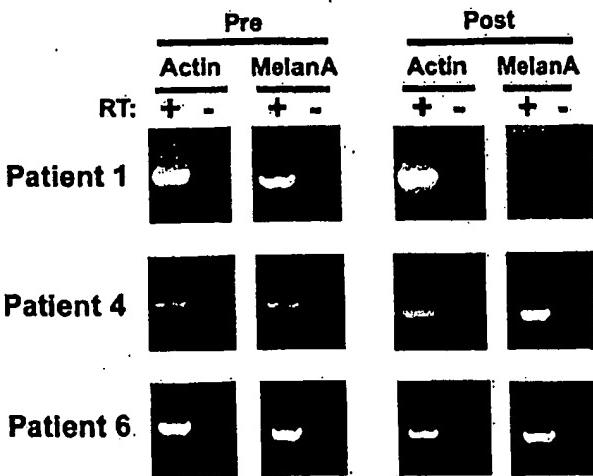


Fig 4. Melan-A expression in tumors that persisted after immunization. Three patients underwent surgical resection of lesions (after discontinuing the study), which were analyzed for Melan-A expression by qualitative reverse transcriptase polymerase chain reaction. Controls were analyzed without reverse transcriptase or with beta-actin primers.

T-cell frequencies using standard assays have been observed in other studies.²⁷

The median overall survival in our study was 12.25 months from treatment initiation, which is greater than the expected 6 to 9 months for this patient population. Although it was a relatively small study and subject to selection bias, most patients were pretreated and had visceral disease, one half of the patients had elevated serum LDH levels, and four patients had treated brain metastases. As has been seen in melanoma patients treated with standard therapies, we found that an elevated serum LDH level was a negative prognostic factor for survival. Whether this is reflective of tumor burden or the metabolic state of the tumor cells that have adapted to an anaerobic environment is unclear.

Some patients developed increases in Melan-A-specific T cells and developed progressive tumor growth despite retained expression of the antigen on posttreatment biopsies. This observation is similar to that seen in murine studies²⁸ and indicates mechanisms of tumor resistance downstream from initial T-cell priming, presumably within the tumor microenvironment. Potential explanations include poor T-cell trafficking to tumor sites, presence of negative regulatory cells, T-cell anergy or death, expression of inhibitory molecules by tumor cells, or downregulation of class I major histocompatibility complex or antigen-processing molecules.^{29,30} Future studies should investigate definable mechanisms of tumor escape that allow tumor cells to resist elimination by antigen-specific T cells *in vivo*.

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Vaccination with Mage-3A1 Peptide-pulsed Mature, Monocyte-derived Dendritic Cells Expands Specific Cytotoxic T Cells and Induces Regression of Some Metastases in Advanced Stage IV Melanoma

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Summary

Dendritic cells (DCs) are considered to be promising adjuvants for inducing immunity to cancer. We used mature, monocyte-derived DCs to elicit resistance to malignant melanoma. The DCs were pulsed with Mage-3A1 tumor peptide and a recall antigen, tetanus toxoid or tuberculin. 11 far advanced stage IV melanoma patients, who were progressive despite standard chemotherapy, received five DC vaccinations at 14-d intervals. The first three vaccinations were administered into the skin, 3×10^6 DCs each subcutaneously and intradermally, followed by two intravenous injections of 6×10^6 and 12×10^6 DCs, respectively. Only minor (less than or equal to grade II) side effects were observed. Immunity to the recall antigen was boosted. Significant expansions of Mage-3A1-specific CD8⁺ cytotoxic T lymphocyte (CTL) precursors were induced in 8/11 patients. Curiously, these immune responses often declined after the intravenous vaccinations. Regressions of individual metastases (skin, lymph node, lung, and liver) were evident in 6/11 patients. Resolution of skin metastases in two of the patients was accompanied by erythema and CD8⁺ T cell infiltration, whereas nonregressing lesions lacked CD8⁺ T cells as well as Mage-3 mRNA expression. This study proves the principle that DC "vaccines" can frequently expand tumor-specific CTLs and elicit regressions even in advanced cancer and, in addition, provides evidence for an active CD8⁺ CTL-tumor cell interaction in situ as well as escape by lack of tumor antigen expression.

Key words: dendritic cells • vaccination • active immunotherapy • melanoma • cytotoxic T lymphocytes

It is now established that the immune system has cells, particularly CD8⁺ CTLs, that can recognize tumor antigens and kill tumors (1, 2). Nevertheless, a major problem is that these T cells are either not induced or only weakly induced, i.e., the T cells are not evident in the systemic circulation. One possibility is that there is inadequate tumor antigen presentation by dendritic cells (DCs),¹ "nature's adjuvant" for eliciting T cell immunity (3). Another is that

tumor-reactive T cells are tolerized by the tumors (1, 4). Melanoma provides a compelling setting in which to pursue a current goal of cancer immunotherapy, the generation of stronger tumor-specific T cell immunity, particularly with CTLs (4). The majority of tumor antigens identified so far are expressed by melanomas (2). Limited antimelanoma CTL responses have been detected (5), and infusions of IL-2 expanded killer cells can lead to rejection of melanoma (6).

Conventional adjuvants promote antibody rather than CTL responses. Therefore, several novel strategies are being explored to induce tumor-specific T cell immunity. DC vaccination is one of these (3). Immature DCs capture

¹Abbreviations used in this paper: CNS, central nervous system; DCs, dendritic cells; DTH, delayed-type hypersensitivity; MCM, monocyte-conditioned medium; RT, reverse transcriptase; TT, tetanus toxoid.

antigens but lack full T cell-stimulatory activity (7). In the presence of appropriate stimuli, such as inflammatory cytokines, the DCs mature. DCs upregulate T cell adhesion and costimulatory molecules as well as select chemokine receptors that guide DC migration into lymphoid organs for priming of antigen-specific T cells. The use of DCs as adjuvants is supported by many animal experiments with primarily mature DCs (3, 8). These studies have shown that the injection of tumor antigen-loaded DCs reliably induces tumor-specific CTL responses, tumor resistance, and in some cases, regression of metastases (3, 8). In the few pilot trials reported so far for humans, immature DCs have been employed (9–11). Scattered tumor responses are reported, but evidence for the induction of tumor-specific CTLs by DC vaccination has not been shown.

We have developed a technique to generate large numbers of homogenous populations of mature and stable DCs from monocytes in the absence of nonhuman proteins (12, 13). We are now exploring the use of these DCs as vaccine adjuvants in humans. Here we provide the proof of the principle by demonstrating that three intracutaneous injections of Mage-3A1 peptide-pulsed mature DCs reliably enhance Mage-3A1-specific CD8⁺ and recall CD4⁺ T cell immunity in heavily pretreated, progressive stage IV melanoma patients with large tumor loads. Expansions of Mage-3A1-specific CTL responses have not been previously detected after Mage-3A1 peptide vaccination in less advanced melanoma patients (14), underscoring the potent adjuvant properties of DCs. As regressions of metastases also occurred upon DC-mediated immunization and were accompanied by CD8⁺ T cell infiltration, we propose that the induced Mage-3A1-specific CTLs are active *in vivo*.

Materials and Methods

Patient Eligibility Criteria

Patients were eligible if they suffered from stage IV (i.e., distant metastases) cutaneous malignant melanoma (1988 American Joint Committee on Cancer/Union Internationale Contre Cancer pTNM staging system) that was not curable by resection and was progressive despite chemo(immuno)therapy. Further inclusion criteria were an expected survival ≥ 4 mo, Karnofsky index $\geq 60\%$, age ≥ 18 yr, measurable disease, HLA-A1 positivity, expression of Mage-3 gene shown by reverse transcriptase (RT)-PCR in at least one excised metastasis, and no systemic chemo-, radio-, or immunotherapy within 4 wk (6 wk in the case of nitrosurea drugs) preceding the first DC vaccination. A positive skin test to recall antigens was not required. Important exclusion criteria were active central nervous system (CNS) metastasis, any significant psychiatric abnormality, severely impaired organ function (hematological, renal, liver), active autoimmune disease (except vitiligo), previous splenectomy or radiation therapy to the spleen, organ allografts, evidence for another active malignant neoplasm, pregnancy, lactation, or participation (or intent to participate) in any other clinical trial. Concomitant treatment (chemo- or immunotherapy, corticosteroids, investigational drugs, paramedical substances) was prohibited. Palliative radiation or surgical therapy of selected metastases and certain medications (acetaminophen/paracetamol, nonsteroidal anti-inflammatory drugs, opiates) to control symptoms were allowed.

Clinical Protocol and Study Design

The study was performed at the Departments of Dermatology in Erlangen, Würzburg, and Mainz, Germany according to standards of Good Clinical Practice for Trials on Medicinal Products in the European Community. The protocol was approved by the Protocol Review Committee of the Ludwig Institute for Cancer Research (New York, NY) and performed under supervision of its Office of Clinical Trials Management as study LUD #97-001. The protocol was also approved by the ethics committees of the involved study centers.

The study design is shown in Table II. All patients gave written informed consent before undergoing a screening evaluation to determine their eligibility. Extensive clinical and laboratory assessments were conducted at visits 1, 5, and 8 (Table II) and consisted of a complete physical examination, staging procedures, and standard laboratory values as well as special ones (pregnancy test, free testosterone in males, autoantibody profile, and antibodies to HIV-1/2, human T cell lymphotropic virus type I, hepatitis B virus, and hepatitis C virus). Patients were hospitalized and examined the day before each vaccination and were monitored for 48 h after the DC injections. Adverse events and changes in laboratory values were graded on a scale derived from the Common Toxicity Criteria of the National Cancer Institute, National Institutes of Health, Bethesda, MD.

Production of the DC Vaccine

During prestudy screening, we tested a small amount of fresh blood to verify that appropriate numbers of mature DCs could be generated from the patient's monocytes (12). Sufficient DC numbers could be successfully generated in all patients, but in some patients the test generation revealed that TNF- α had to be added to assure full maturation. To avoid repetitive blood drawings, we performed a single leukapheresis during visit 2 to generate DCs as described (13). In short, PBMCs from the leukapheresis ($\geq 10^{10}$ nucleated cells) were isolated on Lymphoprep™ (Nycomed Pharma) and divided into three fractions. The first fraction of 10^9 PBMCs was cultured on bacteriological petri dishes (Cat. #1005; Falcon Labware) coated with human Ig (100 μ g/ml; Sandoglobulin™; Sandoz GmbH) in complete RPMI 1640 medium (BioWhittaker) supplemented with 20 μ g/ml gentamicin (Refobacin 10; Merck), 2 mM glutamine (BioWhittaker), and 1% heat-inactivated human plasma for 24 h to generate monocyte-conditioned medium (MCM) for later use as the DC maturation stimulus. The second fraction of 3×10^8 PBMCs was used for the generation of DCs for vaccination 1 and delayed-type hypersensitivity (DTH) test I. Adherent monocytes were cultured in 1,000 U/ml GM-CSF (10×10^7 U/mg; Leukomax™; Novartis) and 800 U/ml IL-4 (purity >98%; 4.1×10^7 U/mg in a bioassay using proliferation of human IL-4R $^+$ CTLL; CellGenix; expressed in *Escherichia coli* and produced under good laboratory practice conditions but verified for good manufacturing practice [GMP] safety and purity criteria by us) for 6 d, and then MCM was added to mature the DCs. MCM was supplemented in patients 04, 06, 09, 11, and 12 with 10 ng/ml GMP-rhu TNF- α (purity >99%; 5×10^7 U/mg in a bioassay using murine L-M cells; a gift of Dr. G.R. Adolf, Boehringer Ingelheim Austria, Vienna, Austria) to assure full maturation of DCs. Mature DCs were harvested on day 7. The third fraction of PBMCs was frozen in aliquots and stored in the gas phase of liquid nitrogen to generate DCs for later vaccinations and DTH tests.

DCs for vaccinations were pulsed with the Mage-3A1 peptide (15) (EVDPIGHLY, synthesized at GMP quality by Clinalfa) as tumor antigen, and as a recall antigen and positive control, tetanus toxoid (TT) or tuberculin (if at visit 1 the DTH to TT in the

Multitest Merieux was >10 mm; both purchased from the Bacterial Vaccines Department of the Statens Serum Institute, Copenhagen, Denmark). The recall antigen was added at 10 µg/ml for the last 24 h, and the Mage-3A1 peptide was added at 10 µM directly to the cultures for the last 8 h (if immunity to recall antigens was strongly boosted, the dose of recall antigen was reduced to 1.0 or 0.1 µg/ml or was omitted for the intravenous DC injections to avoid a cytokine release syndrome). On day 7, mature DCs were harvested, resuspended in complete medium, washed, and pulsed once more with Mage-3A1 peptide (now at 30 µM) for 60 min at 37°C. DCs were finally washed and resuspended in PBS (GMP quality PBS; BioWhittaker) for injection. DCs to be used for Mage-3A1 DTH tests were pulsed with Mage-3A1 (but no recall antigen); DCs that served as negative control in the DTH tests were not pulsed at all. An aliquot of the DCs to be used for vaccinations was analyzed as described (13) to assure that functionally active and mature DCs were generated. The features of the DCs are described in Results. Release criteria were typical morphology (>95% nonadherent veiled cells) and phenotype (>95% HLA-DR⁺⁺⁺CD86⁺⁺⁺CD40⁺CD25⁺CD14⁻ and >65% homogenously CD83⁺⁺).

Immunization Schedule

A total of five vaccinations (three into the skin followed by two intravenously) with antigen-pulsed DCs were given at 14-d intervals (Table II). This design was chosen to explore the toxicity and efficacy of various routes in this trial. For vaccinations 1–3, 3×10^6 DCs were given subcutaneously at two sites (1.5×10^6 DCs in 500 µl PBS per site) and 3×10^6 intradermally at 10 sites (3×10^5 DCs in 100 µl PBS per site). The injection sites were the ventromedial regions of the upper arms and the thighs close to the regional lymph nodes and were rotated clockwise. Limbs where draining lymph nodes had been removed and/or irradiated were excluded. For intravenous vaccinations 4 and 5, a total of 6 and 12×10^6 antigen-pulsed DCs (resuspended in 25 or 50 ml PBS plus 1% autologous plasma) was administered over 5 and 10 min, respectively. Premedication with an antipyretic (500 mg acetaminophen/paracetamol p.o.) and an antihistamine (2.68 mg clemastinhydrogenfumarat i.v.) was given 30 min before intravenous DC vaccination.

Evaluation of Immune Status

Recall Antigen-specific Proliferation and Cytokine Production. PBMCs were cultured in triplicate at two dose levels (3×10^4 and 1×10^5 PBMCs/well) plus or minus TT or tuberculin (at 0.1, 1, and 10 µg/ml) and pulsed on day 5 with [³H]thymidine for 12 h. In all cases, the highest cpm's were obtained with the highest doses of PBMCs and antigen and are shown in Fig. 2. IL-4 and IFN-γ levels were measured in culture media by ELISA (Endogen, Inc.). In a separate plate, staphylococcal enterotoxin (SEA; Serva) was added at 0.5, 1, and 5 ng/ml, and proliferation was assessed after 3 d to provide a positive control for helper T cell viability and responsiveness.

Enzyme-linked Immunospot Assay for IFN-γ Release from Single Antigen-specific T Cells. To quantitate antigen-specific, IFN-γ-releasing, Mage-3A1-specific effector T cells, an enzyme-linked immunospot (ELISPOT) assay was used as described (16). PBMCs (10^5 and 5×10^5 /well) or in some cases CD8⁺ or CD4⁺ T cells (isolated by MACS™ according to the manufacturer, Miltenyi Biotec) were added in triplicate to nitrocellulose-bottomed 96-well plates (MAHA S4510; Millipore Corp.) precoated with the primary anti-IFN-γ mAb (I-D1K; Mabtech) in 50 µl ELISPOT

medium (RPMI 1640 and 5% heat-inactivated human serum) per well. For the detection of Mage-3A1-reactive T cells, the APCs were irradiated T2A1 cells (provided by P. van der Bruggen, Ludwig Institute of Cancer Research, Brussels, Belgium) pulsed with MHC class I-restricted peptides (Mage-3A1 peptide and the HIV-1 p17-derived negative control peptide GSEELRSLY) added at 7.5×10^4 /well (final volume 100 µl/well). After incubation for 20 h, wells were washed six times, incubated with biotinylated second mAb to IFN-γ (7-B6-1; Mabtech) for 2 h, washed, and stained with Vectastain Elite kit (Vector Labs.). For detection of TT-reactive T cells, TT was added at 10 µg/ml directly to the PBMCs (1 or 5×10^5 PBMCs/flat-bottomed 96-well plate). Assays were performed on fresh PBMCs. Spots were evaluated and counted using a special computer-assisted video imaging analysis system (Carl Zeiss Vision) as described (16).

Semiquantitative Assessment of CTL Precursors. The multiple microculture method developed by Romero et al. (17) was used to obtain a semiquantitative assessment of CTLp (precursors) specific for Mage-3A1 peptide. Aliquots of frozen PMBCs were thawed and assayed together. CD8⁺ T cells were isolated with magnetic microbeads (MACS™ separation columns; Miltenyi Biotech) and seeded at 10^4 /well in 96-well round-bottomed plates in RPMI 1640 with 10% heat-inactivated human serum. The CD8⁻ PBMCs were pulsed with peptide Mage-3A1 or the influenza PB1 control peptide VSDGGPNLY (10 µg/ml; 30 min at room temperature), irradiated (30 Gy from a cesium source), and added as an APC population at 10^5 /well together with IL-2 (10 IU/ml final) and IL-7 (10 ng/ml final) in a total volume of 200 µl/well. On day 7, 100 µl fresh medium was substituted, and peptide Mage-3A1 or PB1 (1 µg/ml final) and IL-2 (10 U/ml) was added. On day 12, each microwell was divided into three equal samples to test cytolytic activity in a standard 4-h ⁵¹Cr-release assay on peptide-pulsed (10 µg/ml for 1 h at 37°C) T2A1 cells, nonpulsed T2A1 cells, and K562 target cells, respectively. All of the assays were performed with an 80-fold excess of nonlabeled K562 to block NK activity. Microwells were scored positive if lysis of T2A1 targets with peptide minus lysis without peptide was $\geq 12\%$ and this specific lysis was greater than or equal to twice the lysis of T2A1 targets without peptide plus six as described (18). We aimed at testing 30 microwells of 10^4 CD8⁺ T cells. Therefore, 1/30 positive wells equals at least one CTLp in 3×10^5 (i.e., 30 wells at 10^4 CTLp per well) CD8⁺ T cells (corresponding to $\sim 3 \times 10^6$ PBMCs).

DTH. DTH to Mage-3A1 peptide was assessed by intradermal injection at two sites of each 3×10^5 Mage-3A1 peptide-loaded DC in 0.1 ml PBS. Negative controls were nonpulsed autologous DCs in 0.1 ml PBS and 0.1 ml PBS. DTH to seven common recall antigens (Multitest Merieux) including TT and tuberculin was performed on visits 1, 5, and 8 (Table II).

Assessment and Analysis of Tumor Tissue

For recruitment into the study, Mage-3 gene expression in at least one metastatic deposit had to be demonstrated by RT-PCR as described (14). Accessible superficial skin metastases were removed whenever possible after the vaccinations and subjected to Mage-3 RT-PCR as well as routine histology and immunohistochemistry (to characterize cellular infiltrates).

Statistical Analysis

For analysis of the immune response, pre- and postimmunization values were compared by paired *t* test after logarithmic transformation of the data. Significance was set at $P < 0.05$.

Results

Patient Characteristics

All 13 patients were HLA-A1+, had proven Mage-3 mRNA expression in at least one excised metastasis, and suffered from advanced stage IV melanoma, i.e., distant metastases that were progressive despite chemotherapy and, in some cases, chemoimmunotherapy (Table I). We offered DCs to all patients who fulfilled the inclusion and exclusion criteria, i.e., we did not select for subsets of patients. Two patients (numbers 01 and 03) succumbed to melanoma after two and three vaccinations, respectively. 11 patients received all five planned DC vaccinations in 14-d intervals (Table II) and were thus fully evaluable.

Quality of the Vaccine

All vaccine preparations were highly enriched in mature DCs. More than 95% of the cells were large and veiled in

appearance, expressed a characteristic phenotype by flow cytometry (HLA-DR⁺⁺⁺CD86⁺⁺⁺CD40⁺CD25⁺CD14⁻), and acted as strong stimulators of an MLR at DC/T cell ratios of $\leq 1:300$ (13). Most (mean 80%) expressed the CD83 mature DC marker (19). These features were stable upon removal of cytokines and culture for one to two more days (13). The DCs were pulsed with Mage-3A1 peptide as a tumor antigen and TT or tuberculin as a recall antigen. The latter were internal controls for immunization and possibly provided help for CTL responses (20).

Toxicity

No major (above grade II) toxicity or severe side effects were observed in any patient, including the two patients who were not fully evaluable. We noticed, however, local reactions (erythema, induration, pruritus) at the intracuta-

Table I. Patients' Characteristics, Status before DC Vaccination, and Response to DC Vaccination

Patient code	Sex/Age	Onset stage IV	Previous therapy	Metastases at study entry ^a								Clinical Response	Survival		
				regional		distant									
				skin	LN	Skin	LN	Lung	Liver	Other					
Patients with objective tumor regression															
04	M48	1/98	PCI					1/15 m 30			CNS 2/12 m 30	complete regression of all but 1 lung metastasis, overall progression	10+>9		
06	F61	10/97	CI			3/15		1/20 m 20	2/16			complete regression ^b of 1 lung + 4 s.c. ^c metastases, overall progression	6+>16		
07	F48	6/97	C			1/17					axillary 1/50 m 30	complete regression ^d of 1 lung ^e + 2 s.c. ^f metastases, overall progression	13+12†		
08	M67	11/97	PC		2/25		1/20	2/20	1/30			complete regression ^g of lung + liver + 4 s.c. ^h metastases, overall progression ⁱ	8+3†		
09	F43	5/98	C					1/28			mediastinum 1/5 bone 2/m	Partial regression of 1 lung metastasis, overall progression	4+>11		
12	M54	9/96	CI		3/30		1/20					partial regression of axillary LN metastases, overall progression	28+>9		
Patients without objective tumor regression															
02	F73	5/98	PCI	>50/40				1/28	1/10	m 85	pancreas 1/10	continuous progression	18+5†		
05	F49	10/97	CI		1/15		2/15	1/10				continuous progression	5+>17		
10	M62	8/98	C	6-70		1/30						continuous progression	1+6†		
11	F72	7/98	C		1/25	2/14	3/12			bone 1/m		continuous progression	4+9†		
13	M34	12/97	CI			1/25		1/25	1/25	1/25	esophagus 1/m	continuous progression	12+5†		

Treatment centers: three patients (04, 08, and 12) were treated in Wuerzburg, two in Mainz (patients 10 and 13), and the others in Erlangen.

Pretreatment therapy: PCI, polychemoimmuno. Preceding excisions and radiotherapies are not listed.

Metastases at study entry: the number and diameter of the largest metastases present at study entry are listed (number/diameter in millimeters). m, multiple (>3 metastases).

Survival: (since onset of stage IV and as of 5 August 1999) is listed as months since onset stage IV until study entry + number of months since study entry. ⁱPatient deceased.

^aCNS metastases were regressing at study entry after gamma knife treatment.

^bDeveloped (in part) after study entry.

^cDetermined by autopsy.

^dSudden death from bleeding into CNS metastasis on visit 8.

^eThe regressions of lung metastases in patients 06 and 07 were documented at a staging 3 mo after visit 8. ^fmediast., mediastinum; ^gpancr., pancreas.

Table II. Study Design

Activities	Screen	Leuka pheresis	Vacc. #1 3 Mio s.c. 3 Mio i.d.	Vacc. #2 3 Mio s.c. 3 Mio i.d.	Vacc. #3 3 Mio s.c. 3 Mio i.d.	Vacc. #4 6 Mio i.v.	Vacc. #5 12 Mio i.v.	Final Evaluation
Clinical visit								
Day	-28/-14	-9	+1	+14	+28	+42	+56	+70
Vaccination								
Multitest Meneux	X		X	X	X	X	X	X
DTH to Mage-3A1 peptide-loaded DC			X		X		X	
Recall antigen proliferation		X						X
CTLp analysis		X				X		X
ELISPOT	X	X	X	X	X	X	X	X
Mage-3A1		x	x	x	x	x	x	x
ELISPOT recall antigen								

X, prespecified in the protocol as obligatory; x, optional.

neous vaccination sites that increased with the number of vaccinations. In 9/11 patients, strong DTH reactions (induration >10 mm in diameter) were noted to DCs carrying a recall antigen (Fig. 1). Elevation of body temperature (grade I and II fever) was observed in most (9/11) patients and was also related to pulsing DCs with recall antigen. The most striking example was patient 02, who progressively developed fever (up to 40°C) upon successive vaccinations but did not show a rise in body temperature when TT was omitted for the final (fifth) vaccination. We observed slight lymph node enlargement, clinically in 63% and by sonography in 83% of patients, after the intracutaneous DC injections. Interestingly, these were delayed, being inapparent during the 2 d of monitoring after vaccinations but detected when patients were investigated again the day before the next vaccination (Table II).

Immunological Responses

Boosting of Recall Antigen-specific Immunity. PBMCs that had been frozen before vaccination and 14 d after vaccination 5 were thawed and assayed together, as specified in the protocol (Table II). In most patients, a significant boost of antigen-specific immunity developed to TT (and tuberculin in patient 10) ($P < 0.004$; Fig. 2). Supernatants from the proliferative assays contained large amounts of IFN- γ (mean 1,679 pg/ml, range 846–4,325) but little IL-4 (IFN- γ /IL-4, 317:1). In five patients, we studied the kinetics of the immune response to TT by IFN- γ ELISPOT analysis. We found an increase after the intracutaneous vaccinations ($P < 0.02$) but a peculiar decrease after the intravenous vaccinations ($P < 0.008$; Fig. 3). Thus, comparing recall immunity before and after all five vaccinations (Fig. 2) as prespecified in the protocol (Table II) obviously underestimated the extent of boosting.

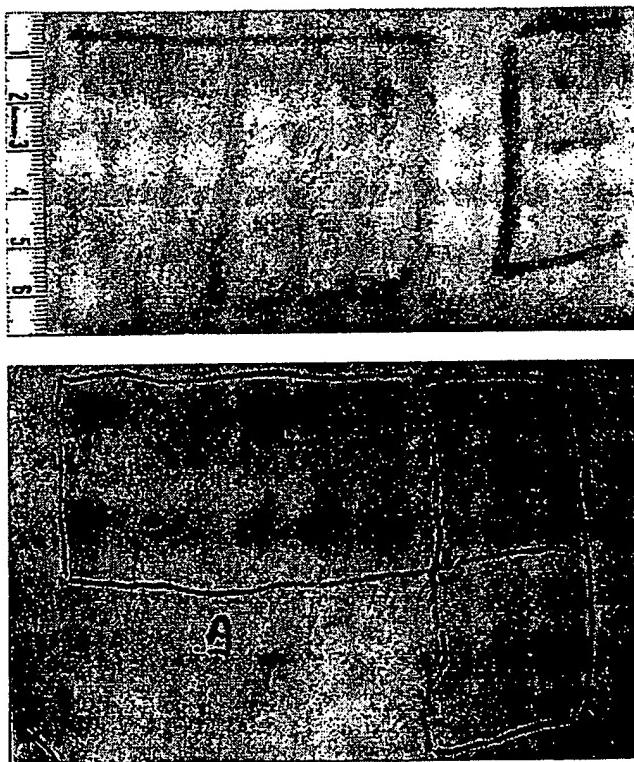


Figure 1. Local reactions to DCs carrying Mage-3A1 peptide and TT at the intradermal and subcutaneous vaccination sites in patient 09 (24 h after vaccination 2; top panel) and 02 (48 h after vaccination 3; bottom panel). Erythema at the 10 intradermal (left) and 2 subcutaneous (right) vaccination sites was followed by induration >10 mm in diameter (with secondary purpura in patient 02). These local reactions represent strong DTH reactions to DCs carrying TT, as such strong reactions did not occur in response to unpulsed DCs or DCs pulsed with Mage-3A1 peptide alone in DTH tests I–III (Table II; reactions not shown).

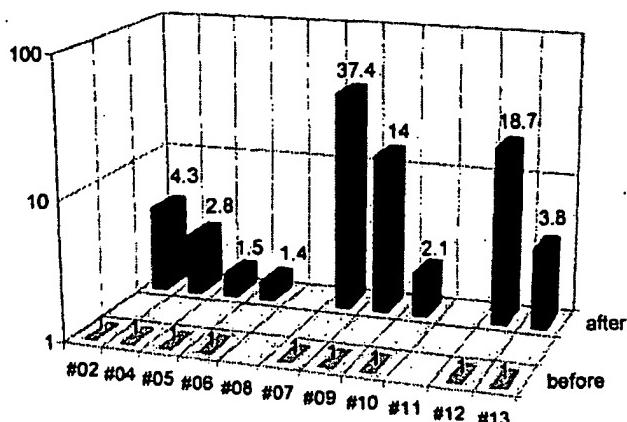
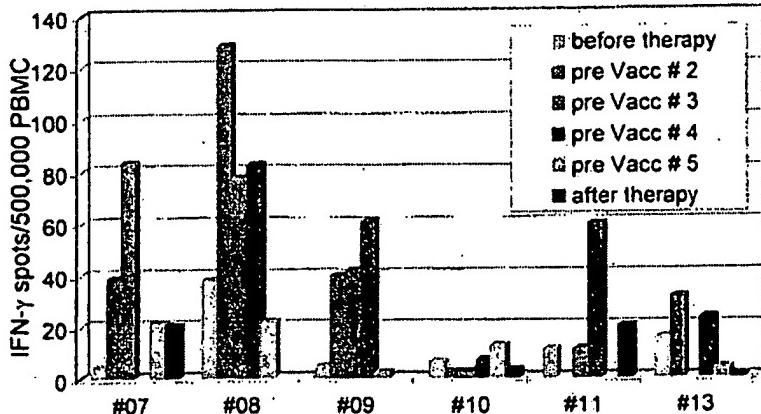


Figure 2. Recall antigen-specific immunity (tuberculin in patient 10; TT in all others) as assayed by antigen-specific proliferation. The cpm values determined after therapy (14 d after vaccination 5) are shown as multiples of pretherapy cpm values. Absolute cpm (cpm with recall antigen minus cpm without antigen) after therapy was 68,917 in patient 02, 85,225 in patient 04, 16,759 in patient 05, 7,913 in patient 06, 16,367 in patient 07, 107,923 in patient 09, 22,790 in patient 10, 4,507 in patient 12, and 1,831 in patient 13 (SEM for all measurements was <20%). Patients 08 and 11 could not be evaluated due to shortage of cells after therapy.

Expansion of Mage-3A1-specific CTLp. Aliquots of PBMCs, frozen before the first and after the third and fifth vaccinations, were thawed at the same time (Table II) and subjected to a semiquantitative recall assay for CTLp (reference 17; Fig. 4). Before vaccination, CTLp frequencies were low or undetectable. In 8/11 patients, we found a significant expansion of Mage-3A1-specific CTLp as indicated by the increase (mean, eightfold; $P < 0.008$) of positive microcultures in the multiple microculture procedure employed for the semiquantitative assessment of CTLp (17). Interestingly, in six patients, the CTLp frequencies were maximal after the three intracutaneous vaccinations ($P < 0.0013$) but then decreased after the two additional intravenous vaccinations in all but one of these patients ($P < 0.026$). Only in 1/11 patients did we observe an increase of CTLp to an irrelevant PB1 influenza peptide that served as a specificity control (not shown).



ELISPOT Analysis for IFN- γ -releasing, Mage-3A1-specific T Cells. We also tried to detect Mage-3A1-specific CTL effectors in uncultured fresh, nonfrozen PBMCs by performing ELISPOT analyses at 14-d intervals on all patients. A significant increase of Mage-3A1-reactive IFN- γ spot-forming cells was apparent only in patients 07 and 09 after the first and second vaccinations, respectively, but the frequency of Mage-3A1-specific effectors was very high (~5,000 and 10,500/10⁷ CD8⁺ T cells; not shown).

DTH Test to Mage-3A1 Peptide-loaded DCs. Tests of DTH to Mage-3A1 peptide-loaded DCs yielded erythema and/or induration (>5 mm diameter) in 7/11 patients (not shown). The results were, however, equivocal due to the frequently observed background to nonpulsed DCs (up to 10 mm in diameter) and the variability from test site to test site.

Clinical Responses

At the end of the trial, i.e., ~2 wk after the fifth vaccination (Table II), we observed temporary growth cessation of some individual metastases, but more intriguingly, in 6/11 patients, complete regression of individual metastases in skin, lymph nodes, lung, and liver (Table I and Fig. 5). Resolution of skin metastases was found in three patients (Table I, patients 06, 07, and 08) and in two of them (06 and 07), it was preceded by local pain, itching, and slight erythema. The six regressing skin lesions of patients 06 and 07 (Table I) were also excised and examined by immunohistology. Clusters of CD8⁺ T cells were seen around and in the tumor, the latter often necrotic, suggesting an immune attack (Fig. 6).

In patients 06 and 08, the metastases excised at study entry (four and two, respectively) proved to be Mage-3 mRNA⁺. However, all of the samples removed at the end (two and six, respectively) were Mage-3 mRNA⁻, suggesting immunoselection for antigen-negative tumor cells. Remarkably, significant infiltration of CD8⁺ T cells was not found in any of these lesions.

Discussion

In deciding on the source of DCs for this phase I trial, we selected mature, monocyte-derived DCs for the follow-

Figure 3. Kinetic analysis of immunity to recall antigens as assessed by TT-specific IFN- γ ELISPOT (SEM for all measurements was <20%). Blood was drawn (see Table II, Study Design) before the first DC vaccination and then every 14 d just before administration of the next DC vaccination (e.g., pre Vacc # 2 means immediately before vaccination 2, i.e., 14 d after vaccination 1), and finally after therapy. Time points at which vaccinations were not performed lack bars. Note the increase after the intracutaneous vaccinations and the decline upon the two vaccinations after intravenous ones. Patient 10, who received tuberculin-pulsed DCs, exhibited no significant change in the TT-specific IFN- γ ELISPOT as expected.

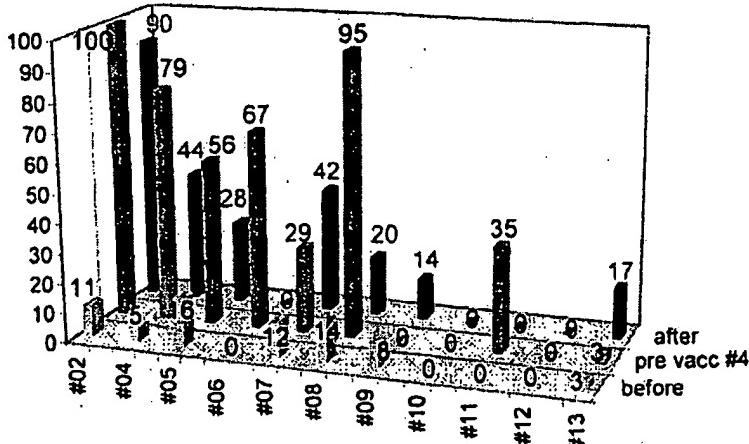


Figure 4. Mage-3A1 CTLp frequency analysis as assessed by semiquantitative recall assay. The y-axes and the numbers above the bars indicate the percentage of positive wells found before vaccination 1, before vaccination 4 (14 d after vaccination 3), and after therapy (usually 14 d after vaccination 5).

ing reasons. Monocyte-derived DCs currently represent the most homogenous and potent DC populations, with several defining criteria and quality controls (12, 13, 21). The method for generating production of these DCs is very reproducible and allows the cryopreservation of large numbers of cells at an identical stage of development (12, 13). Furthermore, these DCs can be produced in the absence of potentially hazardous FCS (12, 13, 21). FCS exposure also leads to large syngeneic T cell responses in culture, so their clinical use (11) might produce nonspecific immunostimulatory effects. Unlike other investigators (9–11), we chose to use mature rather than immature DCs for our first melanoma trial. The DCs that have been used with efficacy in animal experiments were primarily mature (3, 8). Mature DCs are much more potent in inducing CTL and Th1 responses *in vitro* (reference 22 and Jonuleit, H., A. Gieseke, A. Kandemir, L. Paragnik, J. Knop, and A.H. Enk, manuscript in preparation), and the DCs are also resistant to the immunosuppressive effects of IL-10 (23) that can be produced by tumors (24–26). Mature DCs also display an extended half-life of antigen-presenting MHC class I (26a) and class II molecules (27). Finally, mature DCs have a high migratory activity (21) and express CCR7 (28), a receptor for chemokines produced constitutively in

lymphoid tissues (28). Mature DCs, as used in this cancer therapy trial, have recently also been shown to rapidly generate broad T cell immunity in healthy subjects (28).

Mature DCs were loaded with only one melanoma peptide, Mage-3A1, to avoid uncertainties regarding loading of DCs with multiple peptides (11) of varying affinity and off rate. Successful loading was verified with a Mage-3A1-specific CTL clone and ELISPOT analysis (not shown). The Mage-3A1 peptide (15) was selected for several reasons. It is essentially tumor specific (2) and expressed in tumors other than melanoma (2), and the Mage-3A1 epitope is likely a rejection antigen (14). Moreover, the Mage-3A1 CTLp frequency is exceedingly low in noncancer patients (reference 18; 0.4–3 per 10^7 CD8 $^{+}$ T cells) as well as in cancer patients, even after peptide vaccination (14). Thus, any induction or boost of Mage-3A1 CD8 $^{+}$ T cell responses would indicate a significant superiority in the adjuvant capacities of DCs.

DTH assays with peptide-pulsed DCs were carried out as described by Nestle et al. (11) to detect Mage-3A1 immunity (not shown). However, we did not detect unequivocal DTH. This was due to the frequently observed background to nonpulsed DCs (possibly due to cytokine production by DCs) and the noteworthy variability from test site to test site. As Mage-3A1-specific T cells are CD8 $^{+}$

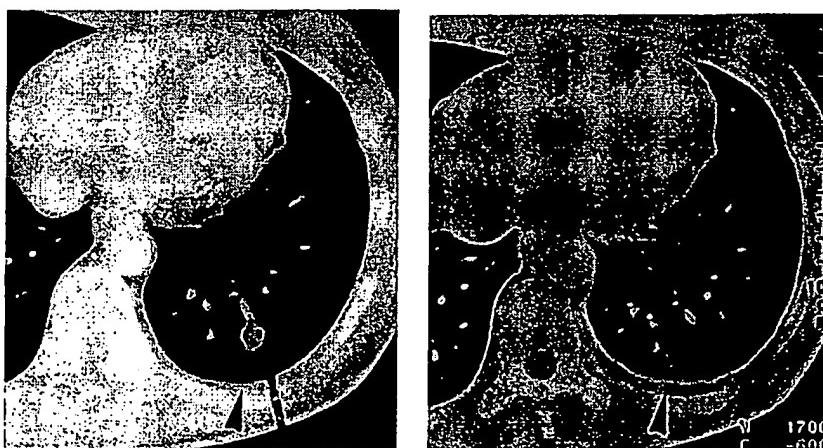


Figure 5. Regression (arrows) of a globular (13 mm in diameter) lung metastasis in patient 07 that was then no longer detectable in serial 6-mm-thick computed tomography scans.

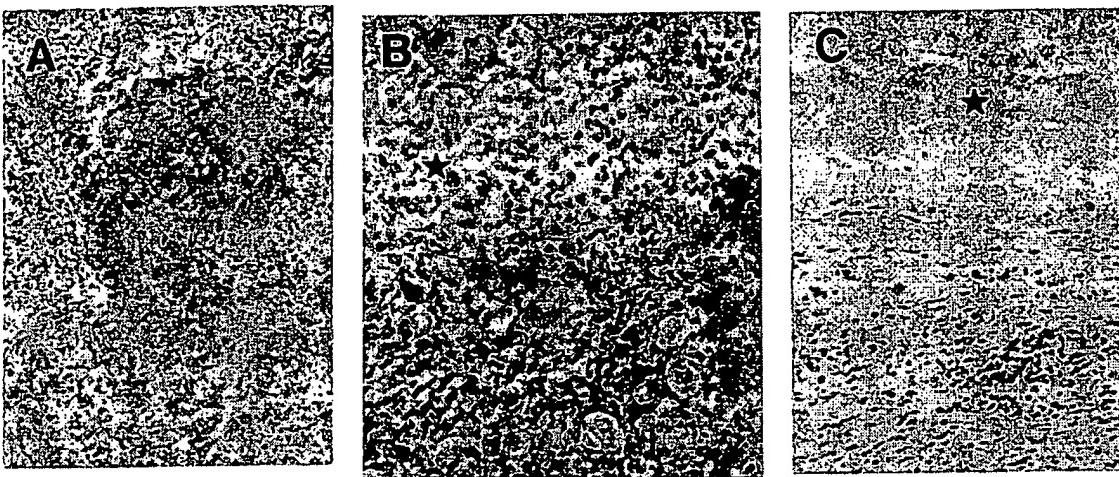


Figure 6. Regressing subcutaneous metastases in patient 06 display a CD8⁺ lymphocytic infiltrate (alkaline phosphatase/antialkaline phosphatase immunohistochemical staining with anti-CD8 mAb) that surrounds (A) and infiltrates (B) the tumor. Areas of damaged (B, ★) and necrotic (C, ★) melanoma cells are obvious in the vicinity of the CD8⁺ T cell infiltrate. The metastasis expressed Mage-3, as demonstrated by RT-PCR (data not shown). Magnifications: A, 100; B, 250; C, 160.

T cells and DTH assays typically detect primed CD4⁺ T cells, we suspect that DTH to MHC class I peptide-pulsed DCs may also for this reason prove not to be a sensitive or reliable way to monitor specific CD8⁺ T cell-mediated immunity.

In contrast, we found sizable expansions of Mage-3A1-specific CTL precursors in PBMCs from a majority (8/11) of patients ($P < 0.008$; Fig. 4). This is an important proof of the principle of DC-based immunization, and it is also significant from the point of view that tumors can induce tolerance or anergy. It is very promising that CTLp expansions can be induced in far advanced and heavily pretreated stage IV melanoma patients. However, active Mage-3A1-specific effectors were generally not observed in ELISPOT assays, except for in two patients with high frequencies ($>5,000/10^7$ CD8⁺ T cells). Perhaps active CD8⁺ effectors were rapidly sequestered in the numerous metastases, as suggested by the biopsy studies illustrated in Fig. 6. An alternative explanation is that looking for effectors in peripheral blood 14 d after a preceding vaccination might simply be too late.

Interestingly, in six patients, CTLp had increased to their highest levels after the three intracutaneous vaccinations ($P < 0.0013$) and then decreased ($P < 0.026$) with subsequent intravenous immunizations (Fig. 4). The decrease in CTLp might be due to emigration of activated Mage-3-reactive CTLs into tissues, tolerance induction, or clonal exhaustion via the intravenous route. We also observed decreased responses to recall antigens in the five patients that we studied before and after intravenous vaccination (Fig. 3). The effect of the intravenous route requires additional study, as it may be counterproductive. In contrast, our results clearly demonstrate that the intracutaneous route is effective, so that the less practical intranodal injection propagated by other investigators (11) does not seem essential. It will, however, be necessary to compare subcutaneous and intradermal routes to find out if one is superior.

We found regression of individual metastases in 6/11 patients when patients were staged 14 d after the fifth vaccination (Table I). This percentage of responses was unexpected in far advanced stage IV melanoma patients who were all progressive despite standard chemotherapy and even chemoimmunotherapy. In the study by Nestle et al. (11), chemotherapy was only given to 4/16 melanoma patients, and objective tumor responses were observed in 5/16. Therefore, we attribute the regressions to DC-mediated induction of Mage-3A1-specific CTLs. This interpretation is supported by the heavy infiltration with CD8⁺ T cells of regressing but not nonregressing (skin) metastases. The observation that all of the metastases in patients 06 and 08 that were excised at the end of the study were Mage-3 mRNA⁻ (whereas those removed at the onset were uniformly positive) suggests immune escape of and selection for Mage-3 antigen-negative tumors. Immune escape might also have been responsible for the lack of tumor response in those nonresponders that had mounted a Mage-3A1-specific CTL response.

After the end of the trial, surviving patients received further vaccinations with DCs and several tumor peptides (Mage-1, tyrosinase, and Mage-3) that were no longer part of the protocol. It is encouraging that 5/11 patients are still alive (Table I) 9–17 mo after study entry, as the expected median survival in patients progressive after chemo(im-muno)therapy is only 4 mo (29, 30). One of the initial responders (patient 06) has recently experienced a complete response and has now been disease free for 2 mo. It is interesting that Marchand et al. (14) have also observed that regressions, once they have started, proceed slowly and may take months to complete.

In conclusion, the use of a defined DC vaccine combined with detailed immunomonitoring provides proof that vaccination with mature DCs expands tumor-specific T cells in advanced melanoma patients. In addition, we have found some evidence for the direct interaction between

CD8⁺ CTLs and tumor cells as well as for escape of antigen-negative metastases. We are convinced that DC-mediated immunization can be intensified further to reveal the presence of expanded populations of effector cells. Efficacy might be increased at the level of the DC, e.g., by optimizing

variables such as DC maturational state, route, dose, and schedule or by improving the short life span of DCs *in vivo* (31, 32); at the level of the T cell, e.g., by providing melanoma-specific CD4⁺ T cell help (33, 34) or IL-2 (35); and by treating patients earlier in their disease course.

We are grateful to all patients for their confidence and cooperation. We appreciate the support of J. Knop, Head of the Department of Dermatology in Mainz. We thank our colleagues H.B.-R. Balda, H. Hintner, F.S.M. Meurer, and C.R. Neumann for referring patients and T.L. Diepgen for statistical analysis. We are particularly grateful to T. Boon and P. van der Bruggen, who helped us in many ways, and to A. Knuth and T. Woelfel for help in establishing the semiquantitative CTL and ELISPOT assays, respectively. We are obliged to the Protocol Review Committee and Office of Clinical Trials Management of the Ludwig Institute for Cancer Research, in particular H.F. Oettgen and E. Hoffman, for their suggestions on improving the protocol and for supervising the trial.

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Immunosuppressive therapy

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Although Cyclosporin A has improved transplant outcome, its use has serious limitations due to its narrow therapeutic window. New approaches to broaden this window exploit alternative drug formulations, pharmacokinetic profiling and new immunosuppressive agents, such as Rapamycin and Brequinar, which act in a synergistic fashion. There is no evidence to suggest that the pharmacological alternative to Cyclosporin A, FK-506, displays a broader therapeutic window, although it may be tenfold more potent. Similarly, despite the specificity of the IgG2a mouse anti-human CD3 monoclonal antibody, it displays a significant range of clinical side effects, delayed therapeutic action and frequently stimulates generation of human anti-mouse monoclonal antibodies. Recent advances in monoclonal antibody technology seek not only to produce antibodies against determinants involved in alloactivation, but also to humanize the antibodies for reduced side effects. The availability of this array of potential agents highlights the need to develop guidelines for clinical trial methodologies to address the unique needs and demands of organ transplantation.

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Introduction

After thirty years of vigorous but relatively unproductive research, the field of immunosuppressive drugs awakened following the approval of two agents that, in contrast to the non-selective drugs Azathioprine (Aza) and corticosteroids, display relatively specific actions on T cells. One of these, the fungal undecapeptide Cyclosporin A (CsA), not only improved clinical outcomes and broadened the clinical settings in which transplants were successful, but also provided a unique tool for dissecting activation mechanisms leading to lymphokine synthesis. Subsequent approval of the other agent, the IgG2a mouse monoclonal antibody (mAb) OKT-3, heralded the use of reagents that bind selective T-cell surface markers to modulate the immune response. The past decade has witnessed striking progress in the development of new pharmacological agents (Fig. 1). One group inhibits lymphokine biosynthesis, FK-506 or signal transduction, Rapamycin (RAPA). A second group is the nucleotide synthesis inhibitors, Mizoribine [1] and RS61443, a morpholinoethyl-ester analog of mycophenolic acid (MPA) [2**], block purine salvage pathways with the generation of guanosine monophosphate, and the quinoline carboxylic acid Brequinar (BQR) blocks the de novo synthesis of pyrimidines [3**]. A third group,

new mAbs, recognizes specific surface epitopes on T cells and antigen-presenting cells (Fig. 2). Immunosuppressive activity has been documented [4-9] with several mAbs that bind various determinants as shown in Table 1. Prolonged graft survival has also been achieved with antibodies, or preferably their F(ab')₂ fragments, directed towards class I [10] or class II MHC antigens. A refinement of mAb technology is the production of immuno-toxins. Ricin α-chain toxin linked to mouse anti-human CD5 IgG1 mAb has been used by Havens (personal communication) to treat steroid-resistant graft versus host disease in human bone marrow transplantation. This array of new agents proffers an unprecedented opportunity to design effective, yet minimally toxic, regimens to improve the outcome of transplantation in man.

Limitations of existing immunosuppressive regimens

Currently, clinical regimens are based upon the use of CsA, the immunosuppressant benefits of which are seriously limited by side effects. In attempts to augment its efficacy, the corticosteroid Prednisone (Pred), Aza,

Abbreviations

- ALG—anti-lymphocyte serum; Aza—Azathioprine; BQR—Brequinar; CMV—cytomegalovirus; CsA—Cyclosporin A; CTL—cytotoxic T lymphocyte; DTH—delayed type hypersensitivity; ICAM—intercellular adhesion molecule; IL—interleukin; LFA—lymphocyte function-associated antigen; mAb—monoclonal antibody; MHC—major histocompatibility complex; MPA—mycophenolic acid; MZB—mizoribine; NFAT—nuclear factor of activated T cells; Pred—Prednisone; RAPA—Rapamycin; TCR—T-cell receptor; Th—T-helper.

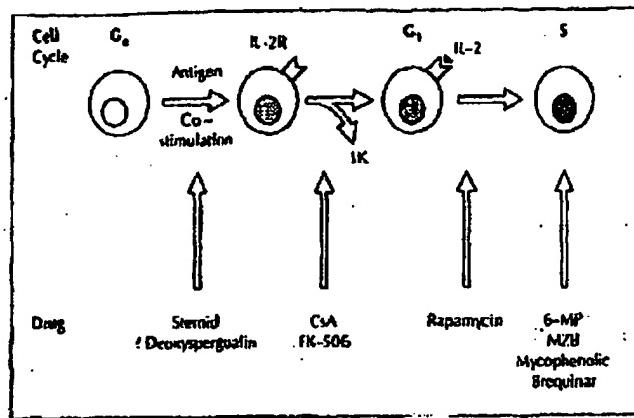


Fig. 1. Classification of immunosuppressive drugs based upon their site of action in the cell cycle. In the first group, corticosteroid, and possibly deoxyspergualin, inhibit antigen-presenting cells. In the second group, Cyclosporin A (CsA) and FK-506 inhibit lymphokine (IL-2) biosynthesis during the G₀ phase and Rapamycin inhibits signal transduction during the G₁ phase. In the third group the nucleoside synthesis inhibitor, Aztreonam (AZD9) and RS61463 (a morpholinooethyl-ether analog of mycophenolic acid, MPA) inhibit purine synthesis pathways leading to the generation of guanosine monophosphate, whereas Brequinar (quinoline carboxylic acid) inhibits the de novo synthesis of pyrimidines. IL, Interleukin; R, receptor.

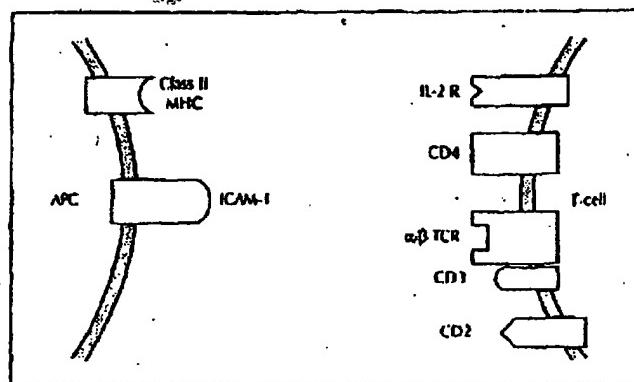


Fig. 2. Epitope targets of monoclonal antibodies. ICAM, Intercellular adhesion molecule; IL, interleukin; MHC, major histocompatibility complex; R, receptor; TCR, T-cell receptor.

Table 1. Determinants recognized by immunosuppressive monoclonal antibodies

Monoclonal antibody	Determinant	Reference
IgM 11089.1A-31	Determinants common to α/β chains of all human T-cell receptors	14
IgG _{2b} 6MA 031	CD4	15
OKT4A	CD7	16
SDZ CHM 300	α chain or α/β complex of interleukin-2 receptor	17
13B.1	Intercellular adhesion molecule-1 (ICAM-1)	18
B8R-1	Lymphocyte function-associated antigen-1	19
253		

equine or rabbit polyclonal anti-lymphocyte sera (ALG) and/or mouse OKT-3 mAbs have been combined in empirical regimens that are often tailored to individual patients. To address one limitation of CsA, nephro- and/or hepatotoxicity during the initial post-transplant phase of induction immunosuppressive therapy, which delays allograft and patient recovery, pharmacokinetic control

programs have been used either to preselect CsA doses [11] or to combine ALG or OKT-3 with AzA/Pred in order to delay treatment with CsA. However, immediate, rather than delayed, administration of CsA to patients displaying good initial renal function avoids the disadvantages of ALG/OKT-3 induction, namely an increased risk of cytomegalovirus (CMV) infection, additional expense and/or delayed hospital discharge awaiting satisfactory CsA levels after treatment with CsA. However, patients at extraordinarily high immunological risk, due to rejection of previous allografts within three months, or with marginally functioning organs may preferably be treated with ALG or OKT-3 induction as a possible means to delay the onset of their first rejection episodes. Two possible alternatives for induction therapy are the CsA analogs that may display reduced nephrotoxicity, namely Cyclosporin G [12], which substitutes norvaline at position 2, and DYM-125 with a hydrophilic substituent at position 9.

To date, no large randomized study has shown that the induction regimen alters the clinical outcome; rather, a small cohort reported by Belitsky *et al* [13] showed no difference between ALG versus initial CsA therapy, the two options for induction therapy. There may be several reasons for this. Firstly, the polyclonal reagents, such as

rabbit or equine Minnesota ALG and anti-thymocyte globulin, regardless of their source, opsonize T cells, leading to their removal from the circulation. This depletion ablates T-cell mediated attack on the allograft. Among other factors that must be considered is that the central intravenous lines required for polyclonal administration may be accidentally contaminated, producing septicemia. Further, there are no indices of the efficacy of the polyclonal sera. Peripheral blood T-cell numbers above the target range of 90–150/ml tend only to be useful reflections of the appearance of human host anti-equine antibodies, not immunological resistance to therapy. The other induction therapy using OKT-3, which covers and/or modulates CD3 epitopes on the T-cell surface, offers the advantages of both peripheral intravenous administration and readily available fluorescence-activated cell sorting (FACS) test for antibody efficacy. The clinician monitors patient peripheral blood lymphocyte T-cell epitopes for (a) cells with exposed CD3 epitopes that were not bound by OKT-3 *in vitro* by their capacity to bind fluoresceinated OKT-3 *in vivo*; (b) the total number of circulating T cells with fluoresceinated anti-CD2, a pan T-cell marker; and (c) the proportion of OKT-3-coated cells detected with a goat anti-mouse IgG reagent. A satisfactory therapeutic effect is observed when the patient has <25% OKT-3⁺ cells *in vitro* and 60–75% CD2⁺ T cells, about 40% of which are coated with mouse IgG which binds to OKT-3. While OKT-3 represents an advance in immunosuppressive therapy, it has several serious limitations: (a) severe first-dose reactions, including chills, fever, myalgias and, in the worst cases, pulmonary edema apparently due to lymphokine release (particularly tumor necrosis factor and interleukin IL-2); (b) longer-term adverse effects such as aseptic meningitis; (c) a delay in the therapeutic effect for as long as 7 days after initiation of treatment; (d) induction of human anti-mouse antibodies, generally of the anti-idiotypic variety, but not uncommonly of broader reactivity; (e) a frequent incidence of rebound re-rejection episodes upon completion of the therapeutic course; and (f) a tendency toward CMV infections in 40% of treated patients. In addition, both polyclonal and mAb reagents may produce excessive immunosuppression, resulting in increased incidences of CMV infection and/or of lymphomas and other neoplasms, as well as allograft thrombosis. Thus, selection of a CSA versus an antibody induction regimen must balance the risks of nephrotoxicity versus that of excessive immunosuppression.

The use of CSA has reduced the risk of acute rejection, but a rational approach to CSA administration is confounded by the tremendous variability between individuals in drug pharmacokinetics and pharmacodynamics [14], which, in turn, generates a fear of irreversible renal injury in case the CSA dose is excessive. Three approaches have been used to address this problem: (a) combining reduced CSA doses with subtherapeutic amounts of AzA [15]; (b) monitoring the parent compound CSA based upon its trough concentration prior to the next drug dose [16]; and (c) adjusting CSA doses prospectively based upon average concentrations calculated from serial measurements of the area under the concentration-time curve

[11]. Since drug absorption presents the greatest variability in pharmacokinetics, attempts have been made to increase CSA bioavailability by co-administration of Vitamin E [17]. In addition, the manufacturer has produced a new micro-emulsion formulation that increases the bioavailability by twofold above that of the existing oral solution or capsule preparation, which show equivalent bioavailability in studies comparing both formulations [18]. The critical issue seems to be the drug concentration in the allograft. While direct intra-arterial infusion has been used for experimental models of renal or cardiac allografts, good drug uptake in man can be achieved by presenting CSA as an aerosol in absolute ethanol when it has a mean particle diameter of 1.2 microns [19].

Optimal use of CSA demands the measurement of drug concentrations/activities at the level of its lymphocyte receptor or target signal transduction molecule(s), which may be calcineurin (an enzyme that may be involved in a common step associated with T-cell and IgE receptor signaling pathways) [20] or the nuclear factor of activated T-cells (NF-AT). However, a major limitation may be the failure of CSA to inhibit lymphocyte activation via the CD28 surface marker [21], an important co-stimulatory pathway that together with T-cell receptor (TCR) stimulation blocks induction of anergy in T-cell clones [22]. Fortunately, rejection episodes under CSA prophylactic therapy tend to be readily reversed by corticosteroid therapy, and the majority of steroid-resistant episodes are overcome with polyclonal ALG and/or OKT-3 therapy. Corticosteroids are believed to represent the Achilles' heel of transplantation because of the wide distribution and pleiotropic effects of the glucocorticoid receptor superfamily found in the cytoplasm. These are DNA binding dimeric transcription factors with a zinc finger structure that recognize enhancer (or negative regulatory) elements bearing the GRE motif (GTACnnnn-GTTCT, where n = any nucleotide). One important negative regulatory element is the AP-1 binding site, normally the focus for fos-jun heterodimers [23]. An alternative approach to the reduction of IL-1 β generation, an action typical of corticosteroids, is to inhibit the enzyme that cleaves the inactive 31 kD precursor between Asp¹¹⁶ and Ala¹¹⁷ to release the 153 carboxyl-terminal amino acids that constitute IL-1 β [24]. Another immunosuppressive effect may be achieved by the upregulation of the synthesis of transforming growth factor- β by steroids [25]. Withdrawal of steroid treatment months to years after the transplant may be successful in patients who did not reject the transplant [26].

Preliminary data suggest that a ten-day course of the IgM mouse anti-human α - β TCR mAb T10B9.1A-31 [4], but not BMA031 (C Grusby, personal communication), not only produces equivalent therapeutic effects to those of OKT-3, but is less toxic in terms of incidence of fever and neurological and respiratory symptoms, as well as of subsequent infections. Furthermore, T10B9 therapy is not associated with as great a rise in serum creatinine during treatment as OKT-3, suggesting a more rapid attenuation of the allo-immune response. However, the repeated use of xenogeneic antibodies during the induc-

tion phase as well as for anti-rejection therapy may be complicated by the development of neutralizing human anti-mouse antibodies.

A major goal of maintenance immunosuppressive therapy is prophylaxis against chronic rejection. To date, not only has CsA/Pred therapy failed to reduce the incidence of this complication from the 8–10% level observed under the Aza/Pred combination, but there is no way to determine if the failure is due to its inherently modest inhibition of B-cell responses or to physicians' tendency to limit CsA therapy to minimal, possibly ineffective, doses in order to mitigate a renal injury. Thus, despite the improvement in initial graft survival, transplants continue to be lost in the longer term, with half-lives of about seven years for cardiac and 11.5 years for renal transplant in humans. A recent study of the effects of immunosuppressive drugs on coronary vascular disease in heterotopic rat cardiac allografts suggests that RAPA particularly, CsA to a lesser extent, but definitely not FK-506, inhibit pathological endothelial and smooth muscle lesions in arteries and arterioles, which seem to be the critical lesions in the progression of chronic rejection [27].

New pharmacological agents

Both the macrolide FK-506 and the undecapeptide CSA interrupt lymphokine synthesis by inhibiting generation of the Ca^{2+} dependent regulatory proteins NF-AT, NFIL-2A, NFIL-2B, and NF- κ B, but not c-fos, which is necessary for IL-2 generation. Presumably, both drugs also affect serine protease gene transcripts, an excellent marker of rejection [28]. The inhibition of cytotoxic T lymphocytes (CTLs), even in the presence of optimal amounts of IL-2, is a prominent effect of CsA [29] and, apparently, FK-506. Despite the assumption that CsA and FK-506 produce similar inhibitory effects, at least three differences have been observed: first, FK-506 displays a flatter inhibition curve than CsA with a wider discrepancy in potency at the 50% inhibition than at the 95% inhibition level; secondly, CsA leads to the generation of suppressive T cells, whereas FK-506 does not; and thirdly, although both drugs inhibit CD4 $^+$ T helper (Th) lymphocytes, which secrete IL-2, only CsA (and not FK-506) permits priming of CD8 $^+$ CTLs [30]. Furthermore, Bretscher and Havrile [31] suggest that CsA switches the immune response to the graft from a delayed type hypersensitivity (DTH) response to an IgG response by inhibiting the Th1 subset with the emergence of the Th2 subset, which actively induces IgG via IL-4 generation and inhibits Th1 cells and DTH via IL-10. Both CsA and FK-506 spare transcription of the down-regulatory lymphokine IL-10. While CsA inhibits transcription of IL-6, this factor is not affected by FK-506.

The coming year should witness publication of a vast array of randomized trials comparing the clinical outcome of liver and renal transplants in patients treated with FK-506 versus CsA. So far, a preliminary non-randomized study of liver recipients showed that FK-506 therapy displays greater neurotoxicity, equivalent nephrotoxicity,

but, possibly, less hypertension than does CsA therapy [32*]. A further claim that corticosteroids do not have to be used with FK-506 cannot be assessed due to two factors: firstly, the protocol stipulated higher Pred doses in the CsA cohort, than those used with FK-506; and secondly, to date, there is no pharmacokinetic analysis of Pred concentrations in CsA versus FK-506 treatment groups in order to exclude a drug interaction. Additionally, the extremely poor results in the initial study, wherein allegedly CsA-resistant patients were converted to treatment with FK-506, actually reflected antagonism between the two drugs caused by (a) an adverse immunological interaction between the two agents that apparently have similar mechanisms of action [33] and (b) competitive pharmacokinetic interactions. Although FK-506 has not yet been shown to achieve clinical results even equivalent to those of CsA, a eventual definition of its relative therapeutic window will depend upon Phase II studies to select well-tolerated drug doses for randomized trials versus CsA therapy.

When a second agent, RS61443, was added in doses of 2500–3500 mg per day to a CsA/Pred regimen, it seemed to reduce the incidence of acute rejection episodes. However, these high doses are likely to produce toxicity, particularly leukopenia and gastrointestinal complaints [2*]. Randomized placebo-controlled trials are underway to assess the efficacy of RS61443 versus Aza added to a CsA/Pred regimen. Other studies are examining the impact of a fourth agent, decoyyspergualin, to potentiate an ALG/Aza/Pred/CsA induction protocol.

The studies that claimed Aza displays pharmacological synergism with CsA failed to utilize rigorous experimental design or data analysis [34]. For instance, both *in vitro* analyses [35] and clinical results demonstrate that Aza acts in an additive manner rather than synergistically with CsA [36]. Similarly, *in vitro* analyses suggest that RS61443 [37], mizoribine [37], and thalidomide [38] also act in an additive manner with CsA. Although initial data suggested that BQR potentiates the effect of CsA [30*], recent experiments document true synergism [37]. However, CsA/RAPA combinations show the most impressive degree of synergy both *in vitro* and *in vivo* [39]. Once Phase I toxicity trials have been completed, it will be possible to assess whether BQR or RAPA displays the synergistic effects with CsA in human transplantation that are evident in rodents and large animal models.

New monoclonal antibody reagents

Second generation mabs are being designed to avoid the severe systemic reactions due to lymphokine release that follow initial doses of OKT-3. For example, the IgG2b anti-human α - β TCR mab UALA (31) used for induction therapy (three 50 mg doses administered on alternate days) delays the onset of first rejection episodes and probably improves one-year graft survival (R Knight and BD Kahan, unpublished data). Similar benefits have been reported with mouse and rat mabs produced against the activation-induced α -chain, or to new epitopes resulting

from the formation of the $\alpha\beta$ complex, of the IL-2 receptor [8].

However, treatment with these antibodies leads to a high incidence of human anti-mouse antibodies, which may attenuate the immunosuppressive effects. Recent work has explored approaches to construct either (a) 'chimeric' antibodies bearing human Fc segments joined to mouse F(ab')₂ fragments, or (b) 'humanized' mAbs with mouse idiotypes inserted onto human IgG isotypes (Fig.3). Chimeric antibodies combine the variable regions of mouse antibodies with human antibody constant regions and, therefore, present fewer foreign amino acid sequences to the host. However, one-third of the structure is still of mouse origin. Furthermore, a clinical trial using a chimeric anti-CD7 mAb not only failed to achieve a superior level of immunosuppression induction, but also increased the incidence of vascular thromboses [7]. The latter effect may have been related to the adhesion of Fc receptors on platelets and polymorphonuclear leukocytes to the human Fc regions, bound to endothelium via mouse epitopes. On the other hand, 'humanized' antibodies combine only the smallest part of a mouse antibody that is required, the antigen combining site, with human variable region frameworks and constant regions. Due to the reduced affinity of 'humanized' antibodies for antigen epitopes, Ci et al. [40] recommended two innovations: firstly, selection of a human framework that is as homologous to the original mouse antibody as possible; and secondly, insertion of key residues from the mouse model into the construct in order to achieve a molecular conformation that is similar to the native idotype. The beneficial effects of chimeric and 'humanized' variants of mouse mAbs will be clarified only by randomized clinical trials.

Two alternative approaches seek to utilize mAbs directed against donor MHC antigens or against co-receptor molecules. In a study of non-human primates,

OKT-4A IgG2A mAbs, which react with the CD4 co-receptor on Th cells, provoked fewer side effects than OKT-3 [6]. In an initial clinical trial of OKT-4A induction therapy (0.2 mg/kg/day), all six patients suffered rejections. These rejections were reversible, but left residual areas of dead tissue resulting from an obstruction of the blood supply in half the renal allografts. Unfortunately, OKT-4A also generated a strong human anti-mouse antibody response (J Barry, personal communication). Experimental animal models are currently being used to determine if antibody efficacy is related to T-cell deletion and is potentiated by simultaneous treatment with an anti-CD8 mAb. On the one hand, Fauffman and colleagues [41] found that depleting anti-CD4 mAbs produced prolonged allo-unresponsiveness toward allogeneic pancreatic islets, an effect that was moderated by simultaneous treatment with anti-CD8⁺ mAbs, suggesting the role of a regulator CD8⁺ cell. On the other hand, Waldmann and colleagues [42] induced tolerance toward mouse heart transplants where the donor and recipient were not matched at MHC level using an anti-CD4 mAb that not only did not deplete T cells but also was potentiated by simultaneous administration of an anti-CD8 mAb.

A second approach to co-receptor molecules is based on the interaction of lymphocyte function-associated antigen (LFA-1) on CTLs with the intercellular adhesion molecule (ICAM-1) on monocytes. Expression of ICAM-1 is up-regulated following lymphokine release, which occurs during acute allograft rejection but not during other pathological events in the kidney [43**]. Prophylactic and therapeutic administrations of a mAb directed against the high molecular weight α -chain of human ICAM-1 alone delayed both the onset and progression of rejection episodes in primate renal allograft models. Using mouse mAbs directed against LFA-1, Stoppa et al. [44] reversed steroid resistant acute graft versus host reactions in man. Indeed, the combination of anti-ICAM-1 and anti-LFA-1 mAbs produced allo-tolerance in mice that were not com-

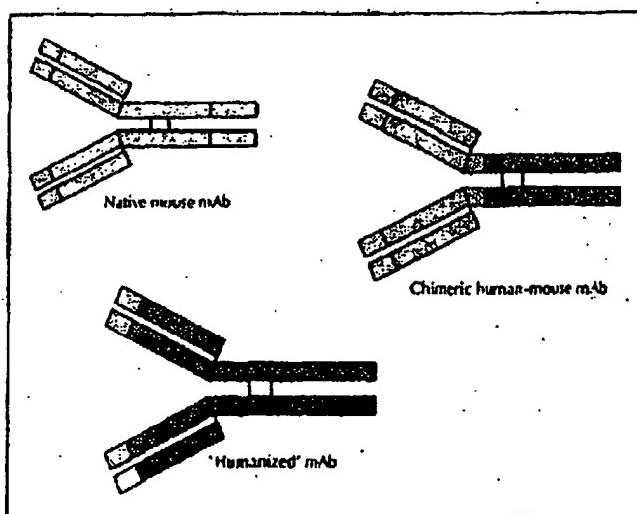


Fig. 3. Types of monoclonal antibody. Chimeric antibodies combine the variable regions of mouse antibodies with human constant regions and, therefore, present fewer foreign amino acid sequences to the host. 'Humanized' antibodies combine only the smallest part of a mouse antibody that is required, the antigen binding site, with human variable region frameworks and constant regions.

patible at the MIC level [45**]. These promising results in animal models using mAbs directed against T-cell and monocyte co-receptors await confirmation in controlled clinical trials.

While clinical interventions to date have focused on using mAbs directed towards surface epitopes important for the effector limb of the allo-immune response, there is increasing evidence that anti-idotypic antibodies, either exogenously introduced or endogenously, spontaneously generated, may regulate the induction of allo-immune responses. A recent study performed by Snider [46] suggested that immunization of hosts with antigen-antibody complexes confers a bias in the epitope, resulting in a less efficient antibody response that shows anti-idotypic properties. This approach represents a particularly fertile ground for clinical exploration.

Cytokine receptor analogs and antagonists

A new group of immunosuppressive agents are the cytokine receptor antagonists. The discovery and initial testing of an IL-1 receptor antagonist has been reviewed by Arend [47]. IgG-stimulated human monocytes naturally produce IL-1 receptor antagonists, a heterogeneous array of glycoproteins of 15–25 kD, depending upon their degree of glycosylation. IL-1 receptor antagonist binds type I, but not type II, IL-1 receptors without activating cells and with considerably less avidity than native IL-1α and IL-1β. Type I IL-1 receptors are present on Th2 cells and fibroblasts; Type II IL-1 receptors are present on B cells, neutrophils, and macrophages. Although therapeutic trials of IL-1 receptor antagonist in rheumatoid arthritis and septic shock suggest some beneficial effects, Pahary et al. [48] failed to observe that IL-1 receptor antagonist inhibited induction of CTls, cutaneous DTH, or T cell dependent humoral antibody responses. They also found that administration of a mAb to type II IL-1 receptor (35F5) was ineffective. Fanslow et al. [49*] recently extended their previous studies, which used constructs of the extra-membranous portion of the IL-1 receptor, by using similar constructs of the IL-1 receptor. In the initial studies, they prolonged heterotopic pinna, neonatal mouse heart allograft survival, but failed to prevent allo-sensitization, as documented by a rapid, secondary-type proliferative response upon *in vitro* one-way mixed lymphocyte reactions. In their recent studies, constructs of IL-1 receptor alone, or in combination with rat anti-mouse IL-1 receptor mAb, induced modest prolongation of heart allo-explants.

Immunosuppressive drug trials

Of the numerous obstacles currently hindering the development of efficacious immunosuppressive regimens, the lack of methodology for clinical transplantation trials is of particular importance. To date, no series of Phase I and II toxicity and dose-finding trials has been conducted in

order to establish a foundation for clinical investigation. The introduction of AzA, steroids, and CsA, as well as the preliminary trials of FK-506, have relied upon empirical approaches. Important obstacles to comprehensive trials include the relatively small numbers of transplant cases, the use of unrefined end-points such as graft and patient survival, and the lack of well-established criteria for the diagnosis and grading of rejection episodes, deficits that obfuscate the use of this event as an intermediate endpoint. In addition, no *in vitro* immune assay predicts or correlates with *in vivo* immunosuppressive efficacy; hence, there is no surrogate immune parameter as a basis of immunosuppressive efficacy and/or for dose extrapolation from *in vitro* systems to *in vivo* conditions.

Since present results with CsA-based regimens yield excellent graft survival, extremely large numbers of patients must be entered into clinical trials to document improved efficacy of a new agent. Even more extensive efforts will be needed to exclude the possibility that the results with the new agent are not actually worse than those obtained with the existent CsA regimen. In light of the presently high success rates, the benefits of any new regimen must be based upon both the potency and the mitigation of side effects, as assessed by quantitative parameters, including glomerular filtration rates. The practice of clinical research in transplantation must proceed to develop principles of rigorous study design and precise analytical tools in order to most expeditiously evaluate the available array of new immunosuppressants described in this review.

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 - Reports that IL-4 is an important endogenous regulator of the allogeneic response *in vivo* and that the specific neutralization of IL-4 is responsible for the inhibition effects of soluble IL-4 receptor observed in the peripheral lymph node system. The results suggest a therapeutic value for IL-4 antagonists alone or in combination with other immunosuppressive regimens.

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APPENDIX 3

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TRANSPLANTATION
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INTERLEUKIN-12 (IL-12)-DRIVEN ALLOIMMUNE RESPONSES IN VITRO AND IN VIVO

REQUIREMENT FOR $\beta 1$ SUBUNIT OF THE IL-12 RECEPTOR¹

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Background. Interleukin-12 (IL-12) mediates its biologic activities via binding high-affinity receptors on T and natural killer cells. Although emphasis has been placed on the requirement for IL-12R $\beta 2$ in IL-12 bioactivity, the role of IL-12R $\beta 1$ is less well defined. The current study evaluated the effects of exogenous IL-12 on alloantigen-specific immune responses and determined the requirement for IL-12R $\beta 1$ in IL-12-mediated alloimmunity.

Methods. The mouse heterotopic cardiac transplant model was employed to evaluate the effects of IL-12 on alloantigen-specific immune responses *in vivo*. In addition, IFN- γ production in mixed lymphocyte cultures (MLC) supplemented with IL-12 was measured to assess the effects of IL-12 on Th1 function *in vitro*. Mice deficient in IL-12R $\beta 1$ (IL-12R $\beta 1^{-/-}$) were used to determine the requirement for this receptor component in IL-12-driven alloimmune responses.

Results. Addition of IL-12 to MLC consisting of wild-type splenocytes enhanced alloantigen-specific proliferative responses and Th1 development. In contrast, IL-12 did not alter these *in vitro* immune parameters in IL-12R $\beta 1^{-/-}$ MLC. Treatment of wild-type cardiac allograft recipients with IL-12 resulted in high concentrations of serum interferon- γ (IFN- γ) and a 10-fold increase in IFN- γ production by recipient splenocytes after restimulation *in vitro*. However, this fulminate Th1 response did not accelerate allograft rejection. Importantly, IL-12 had no effect on serum IFN- γ or in vivo priming of Th1 in IL-12R $\beta 1^{-/-}$ recipients. Finally, administration of IL-12 to WT allograft recipients resulted in a bimodal alloantibody response: antibody production was suppressed at high doses of IL-12, and enhanced at lower doses.

Conclusions. IL-12 markedly enhances alloantigen-specific immune function; however, these exaggerated Th1-driven responses do not culminate in accelerated allograft rejection. Further, these data indicate that IL-12R $\beta 1$ is essential for the enhancement of both *in*

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vitro and in vivo alloimmune responses by exogenous IL-12.

It is well established that interleukin-12 (IL-12*) is a critical cytokine involved in the regulation of Th1- and Th2-mediated immune responses in several experimental models (reviewed in 1 and 2). IL-12 has direct stimulatory and inhibitory effects on Th1 and Th2, respectively (3-6). Further, this cytokine promotes Th1 and inhibits Th2 development indirectly by inducing interferon- γ (IFN- γ) production by activated T cells and natural killer cells (7-12). Th1 have been accepted as key regulators of allograft rejection, in that this cell type promotes both delayed-type hypersensitivity and cytotoxic T lymphocyte responses, which are believed to be the principle terminal effector mechanisms of acute allograft rejection (13, 14). An understanding of the role of IL-12 in graft rejection is just emerging. For example, IL-12 clearly augments alloreactive Th1 development *in vitro* (15). However, the presence of IL-12 is not mandatory for the development of acute cardiac allograft rejection (15, 16). Hence, an important question is whether enhanced Th1 function alters the rejection response. Given the IL-12/Th1 dogma, one would predict that IL-12 would augment alloreactive Th1 function, resulting in accelerated allograft rejection. The present study therefore was designed to test the hypothesis that IL-12-driven Th1 responses would exacerbate cardiac allograft rejection.

IL-12 mediates its biologic effects by interacting with a high-affinity receptor, which consists of at least two cloned components, IL-12R $\beta 1$ and IL-12R $\beta 2$ (17-19). IL-12R $\beta 1$ interacts with the p40 subunit of IL-12, whereas the p35 subunit of IL-12 is believed to bind to IL-12R $\beta 2$ (19, 20). Emphasis has been placed on the necessity for IL-12R $\beta 2$ in IL-12 signaling (21, 22). However, by utilizing IL-12R $\beta 1$ knockout mice (IL-12R $\beta 1^{-/-}$), Wu et al. (23) recently reported that the $\beta 1$ subunit of IL-12R is essential for IL-12-driven proliferation and IFN- γ production by mitogen-activated blasts, natural killer cell lytic activity, and IFN- γ production in response to endotoxin. We have reported that the p40 subunit of IL-12 stimulates alloreactive CD8+ Th1 development both *in vitro* (24) and *in vivo* (15). These observations suggest that

* Abbreviations: Con A, concanavalin A; ELISA, enzyme-linked immunosorbent assay; GVHD, graft-versus-host disease; H&E, hematoxylin and eosin; IFN- γ , interferon- γ ; IL, interleukin IL-12R $\beta 1^{-/-}$, mice deficient in $\beta 1$ subunit of IL-12 receptor; mAb, monoclonal antibody; MLC, mixed lymphocyte culture; WT, wild-type.

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IL-12R β 1 may be needed for alloreactive Th1 development, and that signaling through IL-12R β 1 may be sufficient to mediate IL-12's biologic activity on CD8+ T cells. Hence, the present study employed IL-12R β 1 $^{-/-}$ mice to determine whether β 1 subunit of IL-12R is required for IL-12-induced alloantigen-specific immune responses. To our knowledge, this study is the first to investigate the effects of IL-12 treatment on alloreactive Th1 development *in vivo* and to establish a mandatory role for IL-12R β 1 in IL-12-driven alloimmune responses.

MATERIALS AND METHODS

Mice. Wild-type (WT) C57BL/6 and BALB/c mice between 6 and 12 weeks of age were obtained from Charles River Laboratories (Raleigh, NC). Generation of C57BL/6 IL-12R β 1 $^{-/-}$ mice has been described previously (23). These mice were generated on the 129/Sv background and back-crossed to C57BL/6 mice for five generations, then intercrossed to generate homozygotes.

Medium. The culture medium used in these studies was Dulbecco's minimum essential medium supplemented with 1.6 mM L-glutamine, 0.27 mM L-asparagine, 1.4 mM L-arginine HCl, 14 μ M folic acid, 10 mM HEPES buffer, 1.0 mM sodium pyruvate, 100 units/ml penicillin/streptomycin, 2% fetal calf serum (all obtained from Life Technologies, Grand Island, NY), and 5×10^{-5} M 2-mercaptoethanol (Sigma Chemical, St Louis, MO).

Mitogen-driven cytokine production. To investigate the requirement for the β 1 subunit of IL-12R in mitogen-stimulated IL-10 and IFN- γ production, splenocytes (2×10^6 cells/ml) isolated from naive WT or IL-12R β 1 $^{-/-}$ C57BL/6 mice were incubated for 72 hr with 1 μ g/ml concanavalin A (Con A) (Sigma Chemical). Cultures were supplemented with 1 ng/ml murine recombinant IL-12 (rIL-12) (kindly provided by Dr. Maurice Gately, Hoffmann-La Roche Inc.) to assess the effect of exogenous IL-12 on Con A-stimulated cytokine production by splenocytes of WT and IL-12R β 1-deficient mice. Resulting supernatants were harvested at 72 hr, and the concentrations of IL-10 and IFN- γ measured by enzyme-linked immunosorbent assay (ELISA).

In vitro alloimmune responses. To assess alloantigen-specific Th1 development, splenocytes (1×10^6 cells/ml) isolated from naive WT or IL-12R β 1 $^{-/-}$ C57BL/6 mice were incubated for 5 days with irradiated (5000 rads) BALB/c splenocytes (1×10^6 cells/ml). Where indicated, 1 ng/ml of murine rIL-12 was added to primary mixed lymphocyte cultures (MLC) to assess the effect of exogenous IL-12 on alloantigen-driven Th1 function and to evaluate whether Th1 from IL-12R β 1-deficient mice were responsive to IL-12 stimulation. The concentration of rIL-12 was selected from dose-response experiments in which the amount of rIL-12 needed for maximal enhancement of alloantigen-specific proliferation was 5–10 ng/ml (data not shown). Resulting cell populations were harvested, washed three times, and restimulated (at 1×10^6 cells/ml) with irradiated BALB/c stimulator cells (1×10^6 cells/ml). MLC supernatants were collected after 24 hr (IL-4 and IL-10) or 72 hr (IFN- γ), and cytokine concentrations measured by ELISA.

In addition, splenocyte proliferative response to alloantigens was determined in cultures either left unmodified or supplemented with 1 ng/ml murine rIL-12. WT or IL-12R β 1 $^{-/-}$ C57BL/6 splenocytes (1×10^6 cells/ml) were stimulated for 5 days with irradiated BALB/c splenocytes (1×10^6 cells/ml) in 96-well U-bottom plates (Becton Dickinson, Lincoln Park, NY) in a final volume of 200 μ l (done in quadruplicate). Cultures were pulsed with 0.5 μ Ci/well [methyl^3H]thymidine (ICN, Costa Mesa, CA) for the final 8 hr of the incubation period. [methyl^3H]Thymidine incorporation was assessed on a Wallac 1205 Betaplate scintillation counter (Wallac, Turku, Finland).

Heterotopic cardiac transplantation. Intact BALB/c (H2 d) hearts were anastomosed to the great vessels in the abdomens of WT or

IL-12R β 1 $^{-/-}$ C57BL/6 (H2 b) mice as described by Corry et al. (25). In this model, the transplanted heart is perfused with the recipient's blood and resumes contractions until acutely rejected, which occurs in unmodified WT recipients of this strain combination in approximately 8–9 days (15, 24). Graft function was evaluated by daily abdominal palpation. Myocyte damage and intensity of graft-infiltrating cells were assessed by routine hematoxylin and eosin (H&E) staining of paraffin-embedded sections of transplanted allografts.

Experimental groups. Cardiac allograft recipients were divided into four groups: (1) recipients injected intraperitoneally with 1 mg of anti-CD8 monoclonal antibody (mAb) (hybridoma 2.43, purified by Montana ImmunoTech Inc., Bozeman, MT) on days -2 and -1 before transplantation, (2) animals given daily intraperitoneal injections of murine rIL-12 (0.1 or 1.0 μ g) on days 1–6 after transplantation, (3) recipients injected with a combination of 2.43 anti-CD8 mAb plus rIL-12, and (4) unmodified (no treatment) mice, which served as controls. Depletion of CD8+ cells (<2%) was verified by flow cytometry using anti-CD8:fluorescein isothiocyanate antibody (PharMingen).

In vivo alloimmune responses. To monitor in vivo Th1 development, splenocytes (1×10^6 cells/ml) obtained from allograft recipients were restimulated with irradiated BALB/c stimulator cells (1×10^6 cells/ml), and the concentration of IFN- γ was measured by ELISA. As an additional measure of the in vivo activity of IL-12 on IFN- γ production, sera IFN- γ concentrations in WT and IL-12R β 1 $^{-/-}$ cardiac allograft recipients were measured by ELISA. Further, to assess the effect of IL-12 treatment on B cell function, sera alloantibody (IgM, IgG1, and IgG2a) levels were determined (see below).

Cytokine ELISA. Experimental samples (100 μ l) were added in triplicate to plates coated with 5 μ g/ml rat anti-mouse IFN- γ , IL-4, or IL-10 capture antibodies (PharMingen). Standards were employed by preparing 2-fold dilutions of murine recombinant IFN- γ , IL-4, and IL-10 (PharMingen), with a starting concentration of 25, 2.5, and 10 ng/ml, respectively. After a 1-hr incubation at room temperature, plates were washed three times with 0.05% Tween 20 in PBS. One hundred microliters of rat anti-mouse secondary biotinylated antibodies (1 μ g/ml) (PharMingen) was then added, and plates were incubated at room temperature for 45 min. Plates were then washed three times with 0.05% Tween 20 in PBS, and 100 ml of avidin-peroxidase (Sigma Chemicals) was added. After a 30-min incubation at room temperature, plates were washed three times with 0.05% Tween 20 in PBS, and 100 ml of 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) substrate (Sigma Chemical) was then added to each well. After 20 min, absorbance was determined at 405 nm by an EL 800 microplate reader (Bio-Tek Instruments, Winooski, VT). Sample cytokine concentrations were calculated from a standard curve. The sensitivity of this assay is approximately 300 pg/ml for IFN- γ , 100 pg/ml for IL-4, and 150 pg/ml for IL-10.

Sera alloantibody determination. P815 cells (H2 d) were stained for flow cytometric analysis using dilutions of sera (1:50) obtained from cardiac allograft recipients as the primary antibody, followed by fluorescein isothiocyanate-conjugated isotype-specific anti-mouse IgM, IgG1, and IgG2a secondary antibodies (The Binding Site, San Diego, CA). Data are reported as the mean channel fluorescence determined on a Becton Dickinson FACScan.

Statistics. Statistical analyses in this study were done using a Student's *t* test performed by the program StatView 4.1.

RESULTS

Requirement for IL-12R β 1 in T Cell Responses *in Vitro*

Enhancement of mitogen-driven IFN- γ and IL-10 production by IL-12 requires IL-12R β 1. IL-12 stimulates concomitant production of IL-10 and IFN- γ by activated T cells (15, 26, 27). To determine whether β 1 subunit of IL-12R is required for production of these cytokines, C57BL/6 splenocytes isolated WT or IL-12R β 1-deficient mice were stim-

lated with Con A for 72 hr, and supernatant cytokine concentrations were determined by ELISA. Production of the Th1 cytokine IFN- γ by Con A-stimulated splenocytes isolated from IL-12R $\beta 1^{-/-}$ mice was readily detectable (Fig. 1A), although concentrations were lower than that seen in WT controls (IL-12R $\beta 1^{-/-}$ =1.03 ng/ml vs. WT=5.24 ng/ml). Addition of exogenous rIL-12 significantly enhanced IFN- γ production by mitogen-stimulated splenocytes obtained from WT mice (15.79 ng/ml). In contrast, IFN- γ production by splenocytes from IL-12R $\beta 1^{-/-}$ mice was not altered after the addition of rIL-12 (1.54 ng/ml).

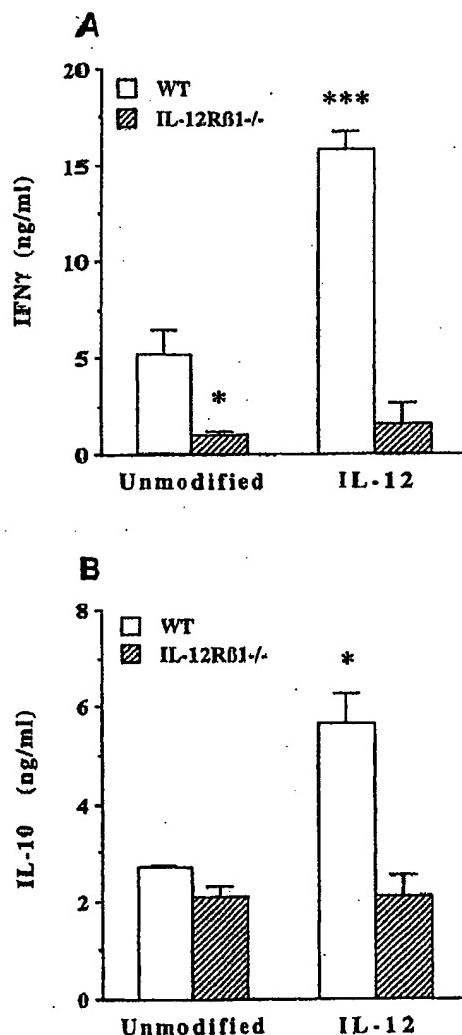


FIGURE 1. Mitogen-driven cytokine production by splenocytes isolated from IL-12R $\beta 1^{-/-}$ mice. Splenocytes (2×10^6 cells/ml) obtained from WT or IL-12R $\beta 1^{-/-}$ C57BL/6 mice were stimulated in vitro with 1 μ g/ml Con A. Cultures were either left untreated or supplemented with murine rIL-12 (1 ng/ml). Supernatants were collected after 72 hr, and the concentrations of IFN- γ (A) and IL-10 (B) were determined by ELISA. Results are expressed as the mean cytokine concentration in triplicate samples \pm SD. Data are representative of three separate experiments. In panel A, *, $P < 0.05$ (WT unmodified vs. IL-12R $\beta 1^{-/-}$ unmodified); ***, $P < 0.005$ (WT unmodified vs. WT IL-12-treated). In panel B, *, $P < 0.05$ (WT unmodified vs. WT IL-12-treated).

The requirement for IL-12R $\beta 1$ in IL-12-driven IL-10 production also was assessed. Splenocytes isolated from IL-12R $\beta 1$ -deficient mice produced similar levels of IL-10 upon Con A stimulation when compared to WT cells (Fig. 1B). rIL-12 enhanced Con A-stimulated IL-10 production by WT splenocytes (2.70 ng/ml vs. 5.65 ng/ml). However, the $\beta 1$ subunit of IL-12R was required for this response, as IL-12 did not affect IL-10 secretion by mitogen-stimulated IL-12R $\beta 1^{-/-}$ splenocytes.

In vitro alloreactive T helper cell development. To evaluate the requirement for IL-12R $\beta 1$ in IL-12-driven alloantigen-specific T cell development, naive splenocytes obtained from WT or IL-12R $\beta 1^{-/-}$ mice were incubated for 5 days with irradiated BALB/c splenocytes in primary MLC, which were either left unmodified or supplemented with rIL-12. Resulting cell populations were restimulated with irradiated BALB/c splenocytes in the absence of rIL-12, and *in vitro* IFN- γ , IL-4, and IL-10 production determined by ELISA (Table 1). Primed WT splenocytes secreted high levels of IFN- γ upon restimulation with alloantigens. Splenocytes obtained from IL-12R $\beta 1^{-/-}$ mice secreted IFN- γ upon restimulation with alloantigens, albeit to a lesser degree than WT cells (WT=21.32 ng/ml vs. IL-12R $\beta 1^{-/-}$ =5.72 ng/ml). The decrease in alloantigen-stimulated IFN- γ production in IL-12R $\beta 1$ -deficient mice was not associated with a decrease in the cells' ability to proliferate in response to alloantigens (Fig. 2), in that [3 H]thymidine incorporation by alloantigen-stimulated IL-12R $\beta 1^{-/-}$ splenocytes was similar to that seen by WT cells (IL-12R $\beta 1^{-/-}$ =13,385 cpm vs. WT=11,441 cpm). In both groups, IL-4, IL-10 (Table 1), and IL-5 (data not shown) were not detected in cultures that were not supplemented with exogenous rIL-12.

As shown in Table 1, exogenous rIL-12 markedly enhanced IFN- γ production by WT splenocytes *in vitro* (21.32 ng/ml vs. 215.13 ng/ml), but failed to augment IFN- γ secretion by cells obtained from IL-12R $\beta 1^{-/-}$ mice (5.72 ng/ml vs. 6.80 ng/ml). Likewise, rIL-12 significantly enhanced WT splenocyte proliferation in the MLC (Fig. 2), but did not alter the proliferative ability of splenocytes isolated from IL-12R $\beta 1$ -deficient mice. Finally, the addition of exogenous rIL-12 to cultures

TABLE 1. IL-12 does not enhance alloantigen-specific Th1 development in IL-12R $\beta 1^{-/-}$ mice *in vitro*^a

Treatment	IFN- γ (ng/ml)	IL-4 (ng/ml)	IL-10 (ng/ml)
Unmodified			
WT	21.32 \pm 0.88	ND	ND
IL-12R $\beta 1^{-/-}$	5.72 \pm 0.24**	ND	ND
IL-12 (1 ng/ml)			
WT	215.13 \pm 13.52***	ND	1.54 \pm 0.21
IL-12R $\beta 1^{-/-}$	6.80 \pm 0.21	ND	ND

^a Splenocytes (1×10^6 cells/ml) obtained from WT or IL-12R $\beta 1^{-/-}$ C57BL/6 mice were incubated for 5 days with irradiated allogeneic splenocytes (1×10^6 cells/ml) in unmodified MLC or MLC supplemented with murine rIL-12 (1 ng/ml). Resulting cell populations were harvested and restimulated with alloantigens for cytokine determination. Supernatants concentrations of IFN- γ (72 hr), IL-4 (24 hr), and IL-10 (24 hr) were measured by ELISA. Results are expressed as the cytokine concentration in triplicate samples (mean \pm SD). Data are representative of four separate experiments. ND indicates not detectable. **, $P < 0.01$, WT unmodified vs. IL-12R $\beta 1^{-/-}$ unmodified; ***, $P < 0.005$, WT unmodified vs. WT IL-12-treated.

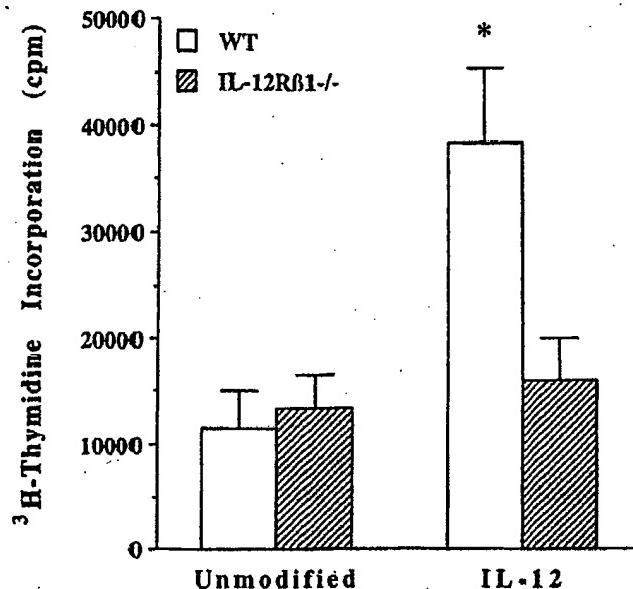


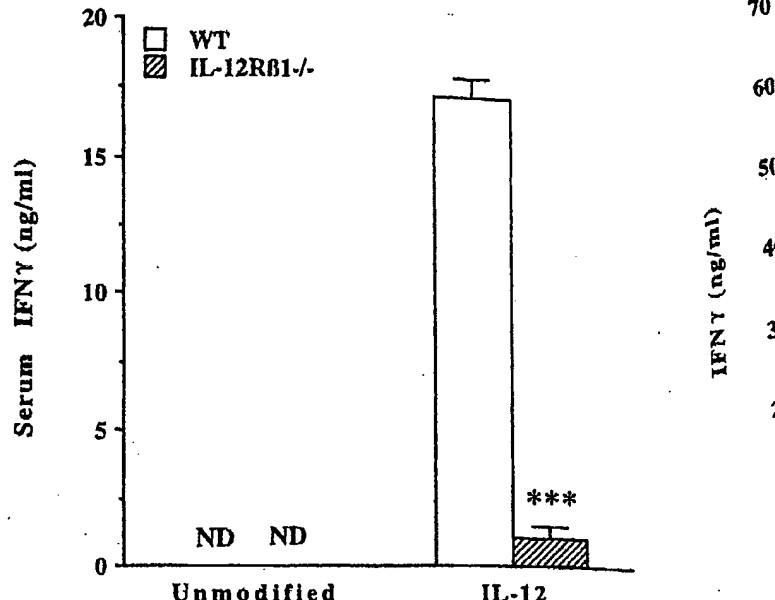
FIGURE 2. The β_1 subunit of IL-12 receptor is required for IL-12-induced stimulation of alloantigen-specific splenocyte proliferation. C57BL/6 splenocytes (1×10^6 cells/ml) were stimulated with irradiated allogeneic BALB/c splenocytes (1×10^6 cells/ml) in 96-well microtiter plates for 5 days. Cultures were pulsed with $0.5 \mu\text{Ci}/\text{well}$ [methyl^3H]thymidine for the final 8 hr of the incubation period, and thymidine incorporation was determined by liquid scintillation spectrophotometry. Results are expressed as the mean cpm in quadruplicate samples \pm SD. Data are representative of three separate experiments. *, $P < 0.05$ (WT unmodified vs. WT IL-12-treated).

stimulated the secretion of IL-10 by alloantigen-stimulated WT splenocytes, but not IL-12R $\beta_1^{-/-}$ cells (Table 1). Collectively, these data indicate that the β_1 subunit of IL-12R is required for the enhancement of several in vitro alloimmune responses by exogenous rIL-12, including increased alloantigen-stimulated T cell proliferation, and IFN- γ and IL-10 production.

Effects of Exogenous IL-12 on Alloimmune Responses in Vivo

Enhancement of serum IFN- γ by IL-12 treatment. To monitor the in situ effects of IL-12 treatment on IFN- γ production in cardiac allograft recipients, serum IFN- γ concentrations were measured on day 7 after transplantation (Fig. 3). In both WT and IL-12R $\beta_1^{-/-}$ allograft recipients, serum IFN- γ was undetectable by ELISA on day 7 after transplantation. Treatment of WT recipients with rIL-12 markedly increased serum IFN- γ in three independent experiments; however, this treatment regimen had little effect on the concentration of serum IFN- γ in IL-12R $\beta_1^{-/-}$ allograft recipients.

Effects of IL-12 on alloantigen-specific Th1 development in vivo. Splenocytes obtained from cardiac allograft recipients were restimulated in vitro with donor alloantigens and supernatant concentrations of IFN- γ were determined by ELISA. This assay detects in vivo primed Th1, in that splenocytes from naive, nontransplanted mice produce minimal or undetectable levels of IFN- γ under these conditions (15, 16, 24). Restimulation of splenocytes from unmodified IL-12R $\beta_1^{-/-}$ allograft recipients with donor alloantigens resulted in the secretion of similar amounts of IFN- γ compared



Treatment In Vivo

FIGURE 3. Treatment of WT, but not IL-12R $\beta_1^{-/-}$ allograft recipients with rIL-12 markedly increases serum IFN- γ . WT or IL-12R $\beta_1^{-/-}$ C57BL/6 mice bearing BALB/c cardiac allografts were either left untreated or given daily intraperitoneal injections of $1.0 \mu\text{g}$ of rIL-12 on days 1–6 after transplantation. On day 7, blood obtained from allograft recipients was pooled and serum collected after centrifugation. Serum IFN- γ was determined by ELISA. Results are expressed as the mean cytokine concentration in triplicate samples \pm SD. Data are representative of three independent experiments. ND indicates not detectable. ***, $P < 0.005$ (WT IL-12-treated vs. IL-12R $\beta_1^{-/-}$ IL-12-treated).

to that seen in WT recipients ($\text{IL-12R}\beta_1^{-/-} = 7.16 \text{ ng/ml}$ vs. $\text{WT} = 6.24 \text{ ng/ml}$) (Fig. 4). Treatment of WT recipients with IL-12 resulted in a 10-fold increase in the production of IFN- γ (59.05 ng/ml). In contrast, IFN- γ production by splenocytes obtained from IL-12R $\beta_1^{-/-}$ recipients treated with IL-12 in vivo was similar to untreated values (9.59 ng/ml), indicating that the β_1 subunit of IL-12R is required for IL-12-mediated enhancement of in vivo sensitization of IFN- γ -producing cells. Further, these results indicate that in vivo Th1 development can occur in a state of IL-12 unresponsiveness.

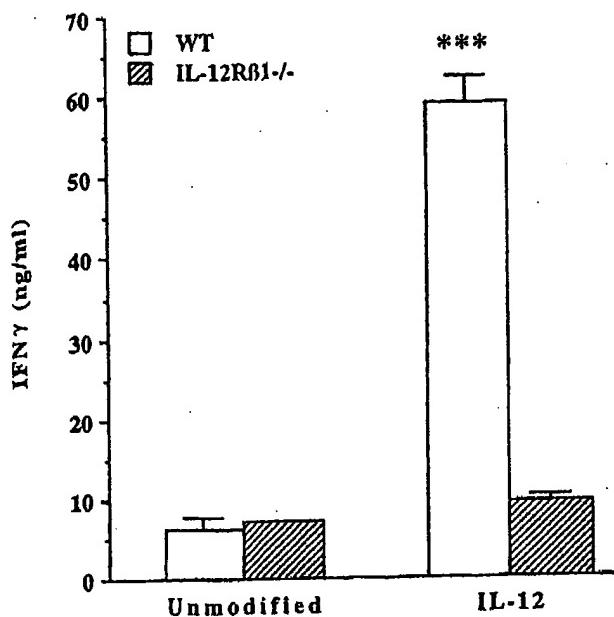
Effects of exogenous IL-12 on cardiac allograft rejection. As IL-12 treatment markedly enhanced Th1 responses in WT allograft recipients (Figs. 3 and 4), one might predict that IL-12 treatment would exacerbate allograft rejection. To test this possibility, cardiac allograft function was monitored by daily abdominal palpation in WT or IL-12R $\beta_1^{-/-}$ allograft recipients bearing BALB/c hearts. Cardiac allograft recipients were either left untreated or injected once daily with $1.0 \mu\text{g}$ of rIL-12. Treatment of WT allograft recipients with this dose of rIL-12 ($n=10$) resulted in symptoms of cachexia including weight loss (mean decrease= $2.0 \pm 0.7 \text{ g}$ in 1 week), ruffed fur, hunched posture, and decreased activity. In contrast, IL-12R $\beta_1^{-/-}$ allograft recipients exhibited no signs of IL-12-induced toxicity.

The mean cardiac allograft survival in unmodified WT recipients was approximately 8 days (data not shown; 15, 24).

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Treatment In Vivo

FIGURE 4. Enhancement of in vivo sensitization of IFN- γ -producing cells by IL-12 requires IL-12R β 1. WT or IL-12R β 1 $^{-/-}$ C57BL/6 cardiac allograft recipients were either left untreated or injected daily with 1.0 μ g of rIL-12 on days 1–6 after transplantation. To assess in vivo Th1 development, splenocytes (1×10^6 cells/ml) obtained from cardiac allograft recipients were restimulated with irradiated BALB/c splenocytes (1×10^6 cells/ml). Supernatants were collected after 72 hr, and the concentration of IFN- γ was determined by ELISA. Results are expressed as the mean concentration of IFN- γ in triplicate samples \pm SD. Data are representative of three separate experiments. ***, $P < 0.005$ (WT unmodified vs. WT IL-12-treated).

Cardiac allografts in IL-12R β 1 $^{-/-}$ recipients were rejected in a similar fashion to that seen in IL-12-deficient mice (15), in that grafts were uniformly rejected by day 7 ($n=8$). As expected, treatment of IL-12R β 1 $^{-/-}$ allograft recipients with rIL-12 had no effect on the tempo of allograft rejection ($n=6$). Interestingly, despite the overwhelming Th1 response induced by rIL-12 in WT allograft recipients (Figs. 3 and 4), treatment of these animals with rIL-12 did not appear to accelerate the tempo of graft rejection when compared to grafts of untreated WT recipients on day 7 after transplantation. For example, 7 of 10 (70%) allografts of WT recipients treated with rIL-12 were still functioning on day 7. A histologic evaluation of these grafts revealed similar parameters of early rejection compared to unmodified WT recipients. Specifically, histology was characterized by diffuse mononuclear cell infiltrates, viable myocytes as evidenced by visible nuclei, and relatively uninvolving vessels (Fig. 5). Hence, rIL-12 treatment did not accelerate the pathologic changes associated with acute rejection.

Phenotype of alloantigen-reactive Th1 in WT allograft recipients treated with rIL-12. To determine the phenotype of Th1 responsive to exogenous rIL-12, WT cardiac recipients were depleted in vivo of CD8+ T cells (Fig. 6). Splenocytes obtained from CD8 depleted cardiac allograft recipients produced markedly less IFN- γ upon in vitro restimulation with irradiated donor splenocytes (WT unmodified = 6.15 ng/ml vs.

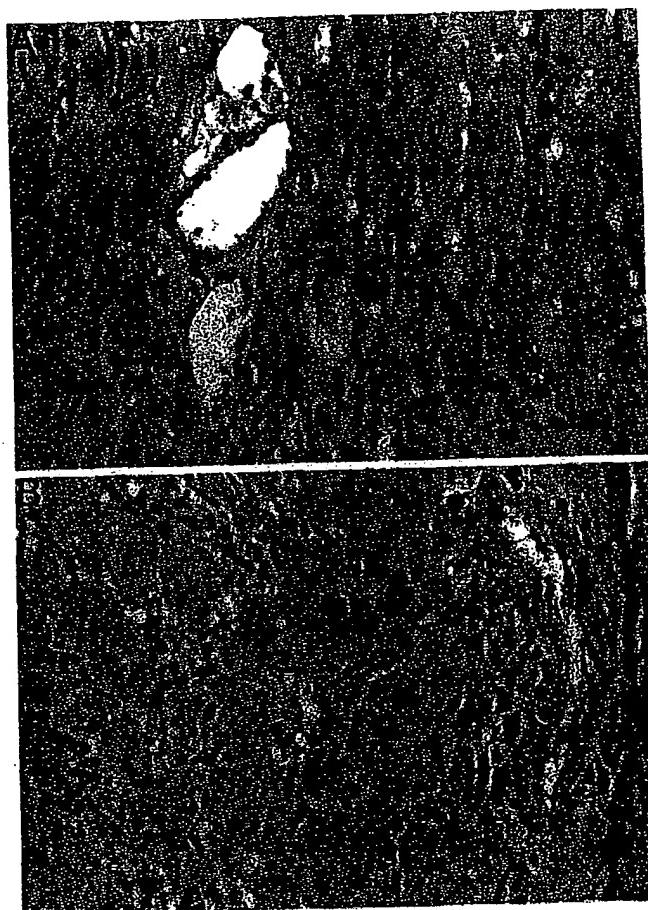


FIGURE 5. Exogenous IL-12 does not exacerbate cardiac allograft rejection. C57BL/6 WT recipients of BALB/c cardiac allografts were either left untreated or injected intraperitoneally with murine rIL-12 (1.0 μ g) on days 1–6 after transplantation. On day 7, allografts were harvested for histologic evaluation. (A) H&E-stained section of allografts from WT recipients left untreated (original magnification, $\times 400$). (B) H&E-stained section of allografts from WT recipients treated with rIL-12 (original magnification, $\times 400$). Note in both experimental groups moderate mononuclear cell infiltrates, and relative health of myocytes and vessels. These characteristics are associated with the early phase of acute rejection before onset of myocyte necrosis and vascular damage, which is observed on days 8 or 9 after transplantation. Results are representative of at least 10 individual transplants for each experimental group.

WT anti-CD8 mAb-treated = 0.52 ng/ml). Similarly, Th1 that develop as a result of IL-12 stimulation in these experiments were predominantly CD8+ T cells (Fig. 6), as depletion of CD8 cells resulted in a reduction in IFN- γ production (WT IL-12-treated = 35.89 ng/ml vs. WT IL-12 plus anti-CD8 mAb-treated = 2.75 ng/ml).

IL-12 Treatment (1.0 μ g/Day) Inhibits Alloantibody Responses

Given our findings that treatment of WT cardiac allograft recipients with rIL-12 resulted in significant augmentation of serum IFN- γ (Fig. 3) and in vivo priming of Th1 (Fig. 4), one would predict that IL-12 treatment should drive IgG2a alloantibody production. To test this possibility, sera alloantibody production was assessed on day 7 after transplanta-

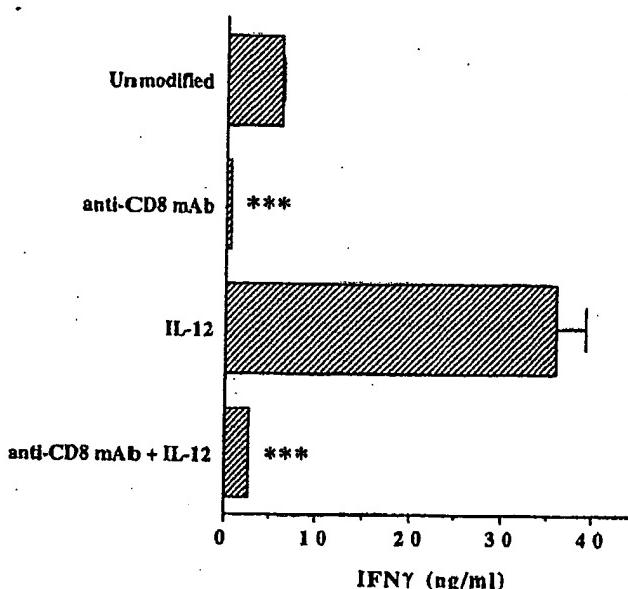


FIGURE 6. Alloantigen-specific Th1 responding to exogenous rIL-12 are CD8+ T cells. In these experiments, splenocytes were obtained from cardiac allograft recipients either left untreated or treated with anti-CD8 mAb, rIL-12, or a combination of anti-CD8 mAb plus rIL-12. Th1 function was assessed by IFN- γ production after a 72-hr restimulation of recipient's splenocytes with irradiated BALB/c splenocytes. Results are expressed as the mean concentration of IFN- γ in triplicate samples \pm SD. Data are representative of three separate experiments. ***, P<0.005 (WT unmodified vs. WT anti-CD8 mAb-treated; WT IL-12-treated vs. WT IL-12 plus anti-CD8 mAb-treated).

tion in WT cardiac allograft recipients either left unmodified or treated once daily with 1.0 μ g of rIL-12. In these experiments, sera IgG2a was undetectable in rIL-12-treated WT allograft recipients at this time point (data not shown). Further, treatment of WT recipients with rIL-12 resulted in reduced sera IgM alloantibody in three independent experiments, compared to untreated WT recipients (Table 2). This observation indicates that high doses of rIL-12 inhibit, rather than enhance, alloantibody production in this model. In contrast, treatment of IL-12R β 1 $^{-/-}$ allograft recipients with

rIL-12 resulted in a slight increase in sera IgM compared to unmodified knockout recipients.

Effects of Low-dose IL-12 (0.1 μ g/Day) on Alloantibody Responses

The reduction in sera IgM in WT allograft recipients after rIL-12 (1.0 μ g/day) treatment (Table 2) may have been caused by anti-proliferative or toxic effects on B cell function caused by rIL-12 and/or IFN- γ . To test this possibility, WT cardiac allograft recipients were treated once daily with 0.1 μ g of rIL-12 and sera alloantibody levels were assessed on day 7 or 8 after transplantation. No alterations in the pathology of allograft rejection was observed in recipients treated with 0.1 μ g of rIL-12 compared to 1.0 μ g (data not shown). Further, treatment of WT allograft recipients with 0.1 μ g of rIL-12 resulted in significantly less IL-12-induced toxicity, serum IFN- γ concentrations in these animals were undetectable by ELISA, and in vivo Th1 sensitization was similar to untreated allograft recipients (data not shown). These results suggested that the 0.1- μ g dose of rIL-12 was ineffective in vivo. However, unlike the higher dose of rIL-12 (1.0 μ g), treatment of WT cardiac allograft recipients with 0.1 μ g of rIL-12 resulted in an increase in sera IgG2a, but not IgG1 at both days 7 and 8 after transplantation (Table 3). Further, IL-12 treatment augmented sera IgM levels at both time points.

DISCUSSION

Bioactive IL-12 exists as a p70 heterodimer composed of p35 and p40 subunits (28, 29). Each subunit of IL-12 interacts with a distinct component of the IL-12R: p40 binds to IL-12R β 1 and p35 interacts with IL-12R β 2 (19, 20). Both receptor subunits are associated with members of the Janus kinase family (30), and therefore may facilitate IL-12-mediated signal transduction. However, most of the attention has been given to IL-12R β 2, which associates with JAK2 (30). For example, recent evidence supports a requirement for IL-12R β 2 expression in IL-12-induced phosphorylation of Stat4 (21, 22). Further, these studies revealed the importance of the β 2 subunit of IL-12R by demonstrating that the unresponsiveness of Th2 to IL-12 in both human (21) and mouse (22) is a result of loss of IL-12R β 2 expression by these cells. These results indicate that the binding of the p40 subunit of

TABLE 2. High-dose rIL-12 inhibits IgM production in vivo^a

	Serum IgM (mean channel fluorescence)	
	Unmodified	IL-12 Treatment
WT		
Experiment 1	37.18	9.85
Experiment 2	33.97	11.39
Experiment 3	78.51	27.37
IL-12Rβ1$^{-/-}$		
Experiment 1	17.36	31.52
Experiment 2	33.83	43.64
Experiment 3	21.67	35.96

^a Serum was obtained on day 7 after transplantation from WT or IL-12R β 1 $^{-/-}$ cardiac allograft recipients. Animals were either left untreated or injected with 1 μ g of rIL-12 once daily. Anti-BALB/c IgM was assessed by flow cytometry using P815 (H2^d) target cells as described under Materials and Methods. Data are reported as the mean channel fluorescence and represent three separate experiments for each group.

TABLE 3. Low-dose rIL-12 augments IgM and IgG2a production in vivo^a

	Mean channel fluorescence		
	IgM	IgG1	IgG2a
Experiment 1 (day 7)			
Unmodified	11.90	2.06	2.58
IL-12	28.94	2.67	12.79
Experiment 2 (day 8)			
Unmodified	31.54	8.53	16.31
IL-12	46.57	9.72	49.00

^a Serum was obtained on day 7 or 8 after transplantation from cardiac allograft recipients either left untreated or injected with 0.1 μ g of rIL-12 once daily. Isotype-specific anti-BALB/c alloantibody was assessed by flow cytometry using P815 (H2^d) target cells as described under Materials and Methods. Data are reported as the mean channel fluorescence. Mean channel fluorescence for isotype controls were 1.65 (IgM), 1.67 (IgG1), and 1.57 (IgG2a).

[IL-12 to IL-12R β 1 is not sufficient to mediate the bioactivity of heterodimer IL-12. However, we have reported that p40 promotes alloantigen-specific CD8+ Th1 development in the absence of heterodimer IL-12 (15). This observation suggests that IL-12 p40 mediates its stimulatory effect through IL-12R β 1 alone, or that IL-12R β 1 associates with a yet unidentified component of IL-12R on CD8+ T cells. These possibilities have not been tested. Additional data are emerging that support a biologic role of p40 interacting with IL-12R β 1 (1). Specifically, p35 knockout mice, which are capable of producing p40 in levels similar to WT mice (31), are less susceptible to infection with *Listeria* and *Cryptococcus neoformans* compared to p40 knockout mice. Hence, one goal of the current study was to assess the role of IL-12R β 1 in alloimmune responses both in vitro and in vivo.

IL-12 is a potent stimulator of in vitro alloantigen-specific Th1 development, in that the addition of IL-12 to MLC consisting of WT responder splenocytes resulted in a 10-fold or greater increase in IFN- γ production (Table 1; 15). Exogenous rIL-12 also markedly augments in vitro Th1 development in mice that are deficient in p35, p40 (15) or both subunits of IL-12 (JR Piccotti and DK Bishop, unpublished observations), indicating that T cells of these mice are equipped with a functional IL-12R. In contrast, IL-12 did not alter MLC IFN- γ production by splenocytes of IL-12R β 1 $^{-/-}$ mice (Table 1). This result illustrates the requirement of β 1 subunit of IL-12R for IL-12-driven Th1 differentiation in vitro. It should be noted that, although IFN- γ production by IL-12R β 1 $^{-/-}$ splenocytes in MLC was reduced compared to WT values (Table 1), this cytokine was readily detectable by ELISA, suggesting that IL-12 is not an absolute requirement for in vitro Th1 responses.

IL-12 is also a key cytokine involved in promoting cell-mediated immune responses in vivo (1, 2). However, what role IL-12 plays in transplant rejection remains unclear. It has been reported that IL-12 has a central role in the progression of acute graft-versus-host disease (GVHD) in mice (6, 32). In these studies, neutralizing IL-12 with a polyclonal anti-IL-12 antibody results in the amelioration of acute GVHD (32) and, conversely, treatment with exogenous IL-12 converts chronic GVHD into exacerbated acute GVHD (6, 32). Further, Williamson et al. (33) have reported that neutralizing IL-12 during the inductive phase of GVHD results in a Th1 to Th2 shift evidenced by a reduction in IFN- γ and enhancement of IL-5 and IL-10 production by Con A-stimulated splenocytes. In contrast to these findings, neutralizing IL-12 in mouse vascularized cardiac allograft recipients promotes intragraft Th2 cytokine (IL-4 and IL-10) gene expression; however, these grafts are rejected in an accelerated fashion compared to untreated recipients (16). Importantly, in vivo Th1 priming is not inhibited by IL-12 neutralization, indicating that Th1 development can occur independent of IL-12 (16). This possibility is further supported by the observation that splenocytes of IL-12R β 1 $^{-/-}$ allograft recipients produce similar concentrations of IFN- γ upon restimulation with donor splenocytes compared to WT recipients (Fig. 4). It does not appear that Th1 development in IL-12R β 1 $^{-/-}$ mice is a result of the interaction of endogenous IL-12 with the low-affinity IL-12R β 2, as treatment of these animals with rIL-12 did not augment in vivo priming of IFN- γ -producing cells (Figs. 3 and 4).

A second hypothesis tested in the current study was treatment of cardiac allograft recipients with IL-12 would accelerate the rejection process as a result of exacerbated Th1-driven immune responses. Administration of exogenous rIL-12 significantly augmented in vivo sensitization of IFN- γ -producing cells in WT cardiac allograft recipients, as evidenced by increased sera IFN- γ (Fig. 3) and enhanced production of IFN- γ by splenocytes after restimulation with donor alloantigens in vitro (Fig. 4). However, this fulminate Th1 response in vivo did not result in anticipated acceleration of graft rejection when compared to untreated control recipients (Fig. 5). It is possible that induction of high systemic levels of IFN- γ results in an inhibition of immune response as a result of IFN- γ 's anti-proliferative properties on effector cell development (34). However, graft survival was not prolonged after rIL-12 treatment in the current study. This observation questions the overall importance of Th1 responses in this experimental model, and suggests that the magnitude of Th1-driven alloimmune response may not correlate directly to the severity of graft rejection. Indeed, Th2-driven immune responses are emerging as potential effector cells of rejection in both human and experimental transplantation (reviewed in 35).

Finally, we examined the influence of rIL-12 administration on allospecific B cell function. In an experimental system in which PVG.RT1^a congenic rats were immunized with an isolated alloantigen, Gracie et al. (36) reported that treatment with murine rIL-12 (1.0 μ g/day for 5 days) after alloimmunization augments levels of allospecific IgG2b and IgG2c, while decreasing IgG1. The authors demonstrated that co-administration of neutralizing anti-IFN- γ mAb abrogated this response, indicating that the enhancement of B cell function by IL-12 was dependent on IFN- γ . When adjusted for body weight, this dose of rIL-12 in the rat is comparable to our 0.1- μ g dose in the mouse. In the current study, treatment of WT cardiac allograft recipients with 0.1 μ g of rIL-12/day for 6 days increased the level of sera IgG2a compared to untreated recipients on day 7 and 8 after transplantation (Table 3). However, administration of 1.0 μ g of rIL-12/day reduced allospecific B cell function, indicated by a decrease in sera IgM (Table 2) and absence of isotype switch to IgG2a. These observations suggest a biphasic response to IL-12 treatment in WT mouse cardiac allograft recipients likely dependent on the concentration of IFN- γ .

In summary, this study illustrates that the β 1 subunit of mouse IL-12R is critical for IL-12-driven alloimmune responses both in vitro and in vivo, and that IL-12R β 2 alone does not transduce IL-12 signaling. These observations are supported by recent reports, which have shown that humans deficient in IL-12R β 1 exhibit severe impairment in their resistance to infections as a result of intracellular pathogens (37, 38). The generation of mice deficient in IL-12R β 2 will provide an important animal model to evaluate whether β 1 subunit of IL-12R alone conveys IL-12 responsiveness in vivo. Specifically, these mice would be useful in determining the mechanism by which p40 subunit of IL-12 enhances CD8+ Th1 development (15, 16). Finally, this study questions the importance of Th1-driven alloimmune responses in cardiac allograft rejection, as exacerbated Th1 responses induced by IL-12 failed to accelerate graft rejection in this model.

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APPENDIX 4

Zinc Inhibits the Mixed Lymphocyte Culture

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ABSTRACT

The mixed lymphocyte culture (MLC) is an established clinical method for bone marrow transplantation, as it serves as an *in vitro* model for allogenic reaction and transplantation. We previously showed that cytokine release into the supernatant is a more specific and sensitive parameter for cross-reactivity in the MLC than the common measurement of cell proliferation. Therefore we tried to find an inhibitor of the MLC *in vitro* with the least side effects *in vivo*, measuring interferon (IFN)- γ as one of the most important cytokines in posttransplant medicine. Earlier studies showed that zinc is an important trace element for immune function with both stimulatory and inhibitory effects on immune cells. We found that slightly elevated zinc concentrations (three to four times the physiological level), which do not decrease T-cell proliferation *in vitro* nor produce immunosuppressive effects *in vivo*, suppress alloreactivity in the mixed lymphocyte culture. In this report we analyzed the mechanism whereby zinc influences the MLC to possibly find a nontoxic way of immunosuppression.

Index Entries: Mixed lymphocyte culture (MLC); mixed lymphocyte reaction (MLR); trace elements; zinc.

INTRODUCTION

The mixed lymphocyte culture (MLC) is a well-established and important tool for determination of compatibility between host and donor

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in transplantation medicine, as it serves as an *in vitro* model for allogenic reaction (1,2). It is common to measure T-cell proliferation in the MLC, but it was recently shown that cytokines are more specific and sensitive parameters for the prediction of a possible graft rejection, as they play a critical role in the posttransplant response (3-5). The TH1-cytokine interferon- γ (IFN- γ) was identified as the most important factor within the cytokine cascade in the MLC (6). It is known to induce cytotoxic T-lymphocytes (CTL) (7) by enhancing the expression of both major histocompatibility complex (MHC) class I and MHC class II molecules (8). The IFN- γ response mainly depends on HLA-DR differences and it therefore well represents reactivity between two individuals in the MLC (9).

In transplantation medicine, cyclosporin A, FK506, and other substances are used to prevent graft rejection. *In vitro* experiments revealed an inhibition of the MLC (10), but, unfortunately, all of these immunosuppressants show a wide range of toxicities *in vivo*, such as nephrotoxicity, neurotoxicity, and, probably, carcinogenicity (11-13). As we are beginning to understand the molecular mechanisms of cyclosporin A and FK506 function better and better, one of the major aims is to find similar substances with less toxicity.

Zinc within the physiological range (12-16 μ M) is an important trace element for immune function (14). Zinc deficiency *in vivo* could be linked to various clinical symptoms such as impaired immune response with regard to decrease in number, differentiation, and function of T-lymphocytes and natural killer (NK) cells as well as decreased activation of monocytes and phagocytosis by macrophages, resulting in a high incidence of bacterial, viral and fungal infections. These symptoms, in the most severe form shown in the hereditary disease acrodermatitis enteropathica caused by malabsorption of zinc, are completely reversible after adequate substitution of zinc (15). On the other hand, high concentrations of zinc (about eight times the physiological level) led to cytotoxic effects with impairment of all T-cell functions, and inhibition of monokine induction by superantigens such as zinc is also important for the binding of some bacterial superantigens to the β -chain of the MHC class II molecule (16,17). Optimal immune-cell function hence requires a well-balanced zinc level.

In the following study, we investigated whether zinc is able to impair alloreactivity in the MLC at concentrations with neither cytotoxic effects *in vitro* nor toxic side effects *in vivo*.

MATERIALS AND METHODS

Preparation of Lymphocyte Cultures

Peripheral blood mononuclear cells (PBMC) were isolated from buffy coats of healthy donors by density centrifugation over Ficoll-Hypaque (Biochrom, Berlin, Germany), washed twice with phosphate-buffered saline (PBS, Gibco, Berlin, Germany) and resuspended in RPMI-1640 medium (Biochrom) supplemented with 10% heat-inactivated fetal calf

serum (FCS, low endotoxin, myoclonal quality; Life Technologies, Eggenstein, Germany), 2 mM L-glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin (all obtained from Biochrom, Berlin, Germany). The cells were adjusted to a final concentration of 2×10^6 cells/mL. Equal volumes of cell suspensions of two donors were seeded in samples to a final volume of 1 mL into pyrogen-free 24-well culture plates (Falcon, Heidelberg, Germany). For controls, 1 mL of the adjusted cell suspension was cultured separately. The cultures were incubated for 5 d at 37°C in a 5% humidified CO₂ atmosphere after addition of the appropriate amount of zinc.

Zinc Preparations

Zinc sulfate (Sigma, Deisenhofen, Germany) was dissolved in sterile water to achieve a zinc stock solution of 10 mM. This solution was further diluted in unsupplemented protein-free medium (PFM, Ultradoma, BioWhittaker) at a ratio of 1 to 2 and then sterile filtered. To achieve the final concentrations, PFM was used. The zinc solution was added to the cultures in a volume of 10% of the final culture volume.

Determination of Cytokines

The culture supernatants were harvested after 5 d and stored at -80°C. The quantification of the cytokine release into the supernatant was performed by enzyme-linked immunosorbent assay (ELISA) technique (for IFN-γ provided by Bender Med Systems, Vienna, Austria). Results were measured in picograms per milliliter at 450 nm using an ELISA plate reader (Anthos Labtec, Salzburg, Austria).

Flow Cytometry

Propidium iodide (PI) staining was performed by using a stock solution of 1 mg/mL (PI, Sigma). Cells (1×10^6 /mL) were incubated with 10 µL of PI stock solution for 20–30 min to allow intercalation of PI in double-stranded DNA. Finally, PI staining was measured at a wavelength of 620 nm in a flow cytometer (Coulter, Krefeld, Germany).

Statistical Analysis

The results are expressed as median values. The significance is taken by Student's *t*-test analysis.

RESULTS

Influence of Zinc on Mixed Lymphocyte Cultures

We harvested the supernatants of zinc-supplemented mixed lymphocyte cultures (MLC) on d 5, proven to be the maximum of the IFN-γ secretion (18). Analyzing IFN-γ release in 20 MLC experiments supplemented with different concentrations of zinc, we found expected amounts of IFN-

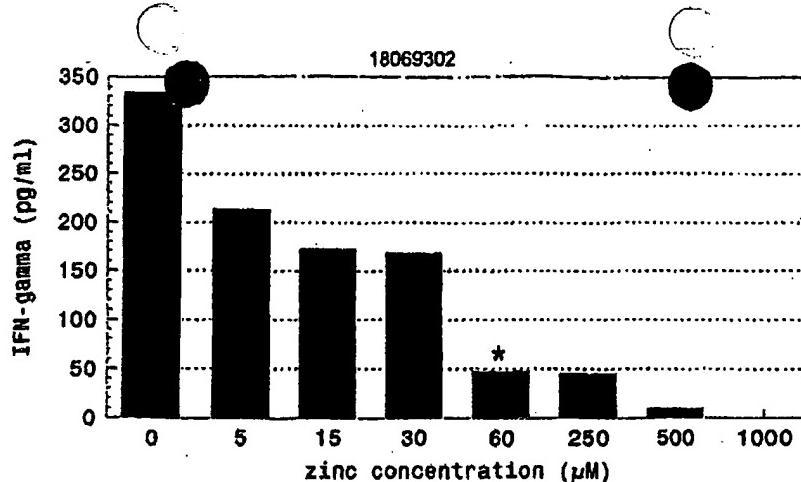


Fig. 1. IFN- γ secretion in the MLC after zinc supplementation. Zinc concentrations up to 1 mM were added to mixed lymphocyte cultures; controls remained unsupplemented. Secretion of IFN- γ in the culture supernatants was determined by ELISA after 5 d of culture. Median values of $n = 20$ experiments are expressed in picograms per milliliter. Significance was calculated by the Student's t -test (* $p = 0.0017$).

γ (334 pg/mL) in the supernatant of control MLC without zinc addition, whereas increasing zinc concentrations led to a dose-dependent reduction of the IFN- γ level. At 60 μM , the IFN- γ production was significantly diminished (48 pg/mL, $p = 0.0017$); at 500 μM , no IFN- γ was detectable (Fig. 1).

In order to prove our hypothesis that this result—that zinc concentrations of 60 μM inhibit the MLC—was the result of a specific effect in the MLC and not to a loss of T-cell vitality, we added zinc concentrations of up to 5 mM to PBMC and measured cell viability by flow cytometry after an incubation time of 48 h. Figure 2 shows that 93.2% of the cells are still vital after addition of 50 μM zinc and 92.3% with medium supplementation of 100 μM zinc compared to controls without zinc addition with 91.3% viability. Zinc concentrations as high as 250 μM causes a reduction of cell survival of 33% (Fig. 2).

For further analysis of possible mechanisms responsible for this inhibition, we preincubated PBMC with 50 μM zinc for 20 min and then cocultured these two populations in the MLC. The results reveal a marked influence of the point of time at which zinc is added to the culture: Preincubation of PBMC led to a greater reduction of IFN- γ than simultaneous zinc supplementation to the MLC (Fig. 3).

DISCUSSION

The human mixed lymphocyte culture (MLC) is an important method to test donor-recipient compatibility in bone marrow transplantation. It could be shown that cytokine release, especially IFN- γ , has a very good predictive value with regard to the transplantation outcome (3), as cytokines play a major role in the generation of an alloreactive immune response and

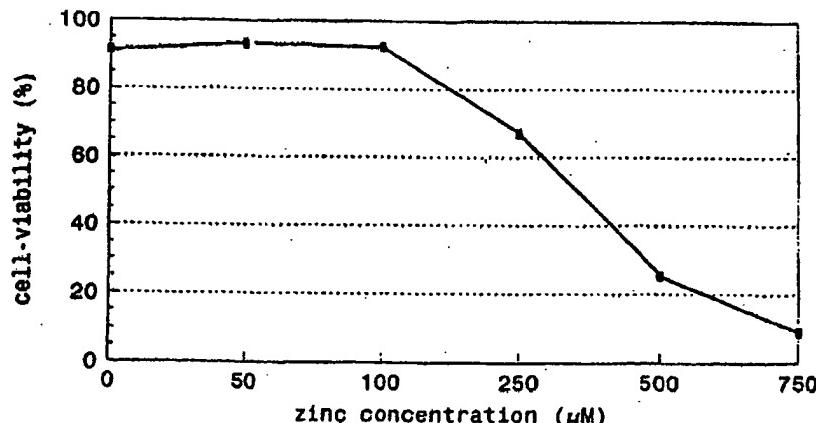


Fig. 2. Viability of PBMC after zinc supplementation. Zinc concentrations of up to $750 \mu\text{M}$ were added to unstimulated PBMC; controls remained unsupplemented. Cell viability was determined by flow cytometry after an incubation time of 48 h. One representative experiment is shown, values are expressed in percent of the total cell population.

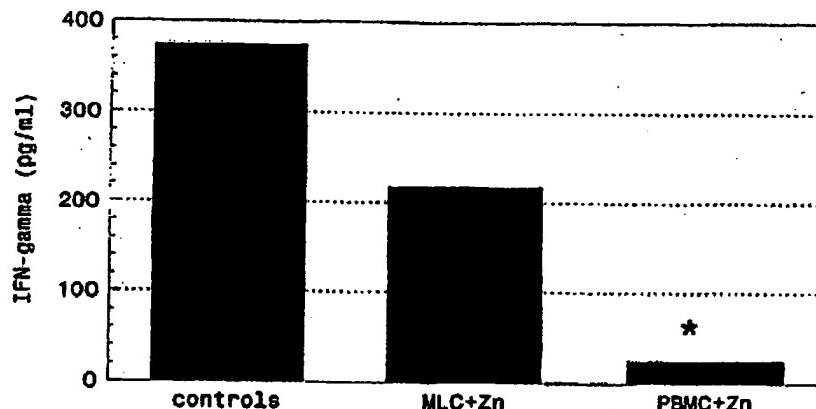


Fig. 3. Effect of preincubation of PBMC with zinc. Zinc in a concentration of $50 \mu\text{M}$ was added to the MLC simultaneously (MLC + Zn); PBMC was preincubated with $50 \mu\text{M}$ zinc for 20 min and then cocultured in a MLC (PBMC + Zn); controls remained unsupplemented. Medians of $n = 10$ experiments are presented. Significance was calculated by the Student's *t*-test (* $p = 0.01$).

for the induction of graft rejection *in vivo* (4,5). Taking this *in vitro* model, it has always been the aim to inhibit proliferation of immune cells in order to find a way to prevent graft rejection in transplantation medicine. Landolfo et al. inhibited T-cell reactivity by the addition of anti-IFN- γ both *in vitro* and *in vivo* (19); others showed a reduced graft rejection rate by repeated injections of monoclonal anti-IFN- γ antibodies in a skin-, heart-, or pancreas-tissue transplantation situation (20-22).

In vivo substances like cyclosporin A or FK506 are broadly applied, as they are capable of prolonging graft survival. In vitro, they show an inhibitory effect on T-cell proliferation in the MLC (10). Yet, all of these therapeutical agents cause major side effects (e.g., nephrotoxicity, neurotoxicity, and others), which lead to a limitation of their use (11-13).

Zinc is an essential trace element with great influence on immune function. The physiological plasma level of zinc ranges from 12 to 16 μM . In our study, we applied zinc concentrations up to 100 μM , which can be reached by pharmacological application of zinc *in vivo* without causing side effects (23).

We found that zinc concentrations of 60 μM , four times the physiological level, inhibit alloreactivity in the MLC. It is unlikely that the reduction of IFN- γ is the result of a loss of T-cell activity, as it could be shown earlier that T-cells are still able to proliferate in medium supplemented with zinc concentrations as high as 100 μM (24). Furthermore, we analyzed the viability of the PBMC by flow cytometry, showing that a concentration of 250 μM is required to reduce cell viability by 33% (Fig. 2).

Increased zinc levels of over 100 μM cause unstimulated human PBMC to release cytokines (25). This stimulatory effect of zinc is only seen in the presence of accessory cells, especially monocytes, as mostly IL-1 proved to be an essential cosignal for T-cell activation by zinc. Higher concentrations of zinc impair all T-cell and monocyte function by inhibition of the IL-1 receptor type I-associated protein kinase (IRAK), thus blocking the intracellular signal transduction pathway at a very early stage (24).

In our study, we applied zinc in concentrations that neither show cytotoxic effects nor reach stimulatory level. Therefore, there seems to be a specific effect of zinc on the responding T-cells in the MLC.

The results of earlier studies proposed an oligoclonal pattern of T-cell stimulation in the MLC similar to T-cell activation by superantigens (3). Furthermore, a highly altered V β repertoire of T-cells infiltrating long-term rejected kidney allografts were described (26). Superantigens bind directly and partially with high affinity to major histocompatibility complex (MHC)-class II proteins, especially to HLA-DR. T-Cell activation is achieved by the formation of a complex of the V β -chain of the T-cell receptor (TCR), the MHC molecule, and the superantigen. This binding is regulated by zinc, as zinc itself does not interact with the MHC molecule directly (27). We previously showed that the HLA-DR and HLA-DQ-molecules have the greatest influence on cytokine release in the MLC and thus on the outcome of a transplantation *in vivo* (9).

There are two main possible explanations for the phenomenon described. First, zinc in the applied concentration could saturate the MHC and, therefore, prevent a binding between TCR and MHC. In order to prove this hypothesis, we preincubated PBMC with zinc and then cocultured these populations. If an extracellular mechanism were actually responsible for the inhibition of the MLC, we would expect no significant difference in IFN- γ secretion in either setting. Preincubation of PBMC

resulted in a markedly lower IFN- γ secretion than the culture of two PBMC populations with simultaneous zinc supplementation to the MLC (Fig. 3), so that it seems more likely that zinc interferes with the intracellular signal transduction in the MLC. Therefore, zinc may regulate the alloreactivity of T-cells and might be an explanation for increased preterm delivery and abortion in zinc-deficient pregnant women (28,29). As mentioned earlier, higher concentrations of zinc are able to block the intracellular signal transduction pathway by inhibition of IRAK. We propose that the stimulation of T-cells by an HLA-different cell population can be blocked by zinc via specific inhibition of phosphorylation processes, leading to a diminished signal transduction in the cell. This results, among other things, in reduced secretion of cytokines, which should lead to less graft rejection *in vivo*. Various protein kinases such as cAMP- and cGMP-dependent protein kinases as well as protein tyrosine kinases are involved in zinc-induced cell stimulation and zinc also influences gene expression of different immunologically relevant transcription factors such as nuclear factor (NF)- κ B and metallothionein transcription factor (MTF-1) as well as others. Which alteration of signal transduction zinc exactly inhibits the MLC remains the subject of further investigation. Because the MLC is inhibited by very low zinc concentrations, this inhibitory effect seems to be a specific pathway.

In conclusion, zinc could become an immunosuppressant in transplantation medicine without toxic side effects, which still leaves the immune system with the ability for phagocytosis. The infection rate will therefore be reduced compared to current immunosuppression. However, this has yet to be proven in *in vivo* transplantation models.

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Antibacterial Effect of Borage (*Echium amoenum*) on *Staphylococcus aureus*

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Borage (*Echium amoenum*) is a large annual plant of the Boraginaceae family, which grows in most of Europe and in northern Iran. The borage flower is used as a medicinal herb in France and other countries. Iranian borage is used in traditional medicine for infectious diseases, flu and as an anti-febrile. We tested the aqueous extract of borage dried flowers in vitro for its antibacterial activity. The extract showed concentration-dependent antibacterial activity against *Staphylococcus aureus* 8327. This activity was heat resistant, but the activity of freeze-dried extract gradually diminished during a 90-day period. The traditional use of Iranian borage flowers for infectious diseases and for controlling fever appears to be justified.

Key Words: Borage, *Echium amoenum*, antibacterial activity, *Staphylococcus aureus*.

Borage (*Echium amoenum*) is a large hairy annual herb that is a member of Boraginaceae family [1]. It grows in most of Europe, in the Mediterranean region, and also in northern parts of Iran. The flowers are bright blue and star-shaped and the fruit consists of four brownish-black nutlets. Borage flourishes in ordinary soil and may be propagated by division of rootstocks and by cuttings of shoots in sandy soil in a cold frame in summer and autumn or from seeds sown in good light soil from mid of March to May [2].

The flowers and the leaves of borage are used medicinally in France as an antifebrile, anti-depressive, for the treatment of stress and of circulatory heart diseases, for pulmonary complaints, as a poultice for inflammatory swellings [3,4], as a diuretic (due to potassium nitrate), as a laxative, emollient and demulcent (due to the mucilage), and recently as a possible protective factor against cancer [5]. The plant constituents

have been isolated by different investigators; they include gamma-linolenic acid (GLA), alpha-linolenic acid (ALA), delta6-fatty acyl desaturase, delta8-sphingolipid desaturase [6], pyrrolizidine alkaloids, mucilage, resin, potassium nitrate, and calcium salt combined with mineral acids.

We tested an aqueous extract of dried borage flowers in vitro for its antibacterial activity against *Staphylococcus aureus* 8327.

Materials and Methods

Plant and extract

The borage found in Iran is *Echium amoenum* (F.M.), which is different from the borage grown in Europe, *Borago officinalis* L. (Boraginaceae). Dried borage flowers were collected from Ardebil province, in northern Iran in mid August. Cold aqueous extract (pH 5.8) of dried *E. amoenum* flowers (5%, w/v) was used in all the experiments. Dried flowers (15 g) were steeped for 6 h at 4°C in 300 mL distilled water, with constant stirring. The material was centrifuged and the supernatant was filter-sterilized and then freeze-dried.

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Anti-bacterial effect of extract

Staphylococcus aureus 8327 was obtained from Tehran University of Medical Sciences, Faculty of Health, Iran. Nutrient broth (NB) containing 5 g peptone (Disco), 5 g NaCl and 3 g beef extract in 1 liter of distilled water (pH 7.5) was used as a culture medium. Anti-bacterial activity of the extract was determined by agar-well diffusion, disc diffusion [7], and the minimum inhibitory concentration (MIC) methods [8]. In the agar-well diffusion method, 9 mm diameter wells were prepared on agar containing 0.5 mL of bacteria (2×10^{11} cells/mL). Freeze-dried extract was diluted 1:20 and different concentrations (1.25 to 10 mg) were added to the wells. In the disc diffusion method, paper discs were soaked in extract solutions and were placed on the bacteria. After 24 h at 37°C, the inhibition zones were measured.

To determine MIC, 5 mL medium was added to six tubes. In the first tube 5 mL extract (1:20 dilution, 50 mg/mL) was added and after mixing 5 mL was removed and added to the second tube; the dilutions continued for all the tubes. Then, 14 mL medium and one mL bacteria suspension were added and the tubes were incubated for 24 and 48 hr at 37°C.

Chromatography

Thin layer chromatography was used to identify the active ingredients of the aqueous extract. Chromatography was performed for 15 h using butanol:acetic acid:distilled water (5:1:4) solvent on a Whatman #1 filter paper. Spots were stained with ninhydrin (to detect amino acids and flavonoids), bismuth iodine, 3% ferric chloride (to detect esters of carboxylic acids and anhydrides), and with Fehling's A+B solution.

Results and Discussion

To determine the antibacterial effect of borage flowers, an aqueous extract was prepared. To get the best aqueous extraction, distilled water with three

different pHs, 5.8, 7.0 and 8.5, was used, and about 8.2, 6.8 and 7.0 g lyophilized powder were obtained, respectively, from 15 g dried flowers. The agar-well diffusion method with 1:20 dilution of these different extracts gave inhibition zone diameters of 10, 7 and 6 mm at pH 5.8, 7.0 and 8.5, respectively. Therefore, pH 8.5 was selected for extraction. Table 1 shows antibacterial effects of various concentrations of borage extract with two different methods. The activity was bactericidal, since incubation of the inhibition zone for one week did not show any growth of bacteria.

The inhibitory effect of extract was not due to the pH of the extract, since extract with all three pHs of 5.8, 7.0 and 8.5 had antibacterial activity, and the control pH had no effect. These data indicate that the antiviral activity of the extract is due to the borage component. The MIC of extract on *Staphylococcus aureus* 8327 after 24 and 48 h was determined to be 6.2 mg/mL. Lower dilutions had no anti-bacterial effect.

The anti-bacterial activity of the extract was heat resistant. Autoclaving the extract at 110°C for one hour did not eliminate its antibacterial activity, and the effect was similar to that of the extract that was filter sterilized. When 200 µL of 1:20 dilution of extract was used in 9 mm diameter wells, in both cases the inhibition zones were 12 mm. The stability assay showed that the anti-bacterial effect of the freeze-dried extract diminished during 90 days storage at 4°C (Figure 1); and the activity of the working solution was diminished after one week at 4°C.

These results indicate that the traditional use of the Iranian Borage flower for infectious diseases and for antifebrile activity may be justified. Borage syrup was thought not only to be good for fever, but also to be a remedy for jaundice, itch and ringworm [2]. Also, we have found that borage extract has anti-viral activity (unpublished data). Already, several components, such as linolenic acid, delta6-fatty acyl desaturase, delta 8-sphingolipid desaturase [6], and pyrrolizidine alkaloids, have been isolated and characterized. In the chromatography experiment, when filter paper was stained with different reagents (Table 2), several spots with different colors and different Rf were obtained. These spots show that the aqueous extract has amino

Figure 1. Stability of antibacterial activity of Borago extract during 90 days by agar-well plate. Freeze-dried extract was prepared (5% W/V) and 0.2 μ m was added to the wells.

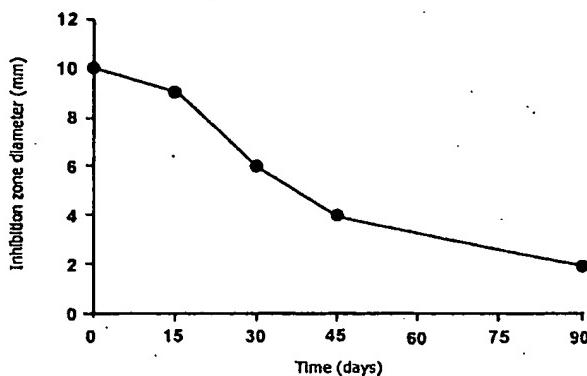


Table 1. Antibacterial effect of Borage flower extract on *Staphylococcus aureus*

Method	Extract (mg)	Inhibition zone diameter (mm)
Disc diffusion	4	8
	1	4
	0.5	0
	0.1	0
Agar-well diffusion	10	10
	5	4
	2.5	0
	1.25	0

Table 2. Thin layer chromatography of aqueous Borage extract on Whatman #1 paper using butanol:acetic acid:water

Reagents	Rf
Ninhydrin	0.13, 0.18, 0.23, 0.38, 0.48, 0.63 (all purple)
Ferric chloride (3%)	0.24 (white), 0.61 (brown)
Fehling (A+B)	0.21 (yellow)
Bismuth iodine	No spot

acids, but no alkaloids. No antibacterial activity was identified when these spots were placed on bacterial culture. It seems that the materials in these spots are not enough for anti-bacterial activity; however, more data are needed to determine the active anti-bacterial components of the extract

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ECHIUM AMOENUM STIMULATE OF LYMPHOCYTE PROLIFERATION AND INHIBIT OF HUMORAL ANTIBODY SYNTHESIS

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ABSTRACT

Background: In Iranian traditional medicine, *Echium amoenum*, is used as a native medicinal plant.

Objective: To investigate the effect of *Echium amoenum* extract on humoral and cellular immune response.

Methods: Hydroalcoholic extract of *Echium amoenum* was prepared and the proliferative responsiveness of peripheral blood and thymic lymphocytes to mitogen and alloantigen was determined by lymphocyte proliferation assay and two-way mixed lymphocyte reaction, respectively. Humoral antibody production to sheep red blood cell (SRBC) in mice was investigated by multiple intraperitoneal administration of the extract (1-100 µg/ml) 24 and 48 hours before and after immunization of a group of mice with SRBC. The primary and secondary antibody titers to SRBC were determined by hemagglutination assay.

Results: A strong increase in allogenic response was obtained at 10 µg/ml of the extract (stimulation index of 1.81, $p < 0.01$). *Echium amoenum* showed no direct stimulatory activity on either lymphocytes or thymocytes in proliferation assay. However, the extract at concentrations of 50-400 µg/ml showed a co-stimulatory effect on mitogenic lymphocyte proliferation. The highest stimulation index was obtained at concentration of 200 µg/ml (1.44, $p < 0.02$). Despite these immunostimulatory effects, the extract showed a significant dose related decrease in the humoral antibody response ($p < 0.05$). The mean antibody titer log₂ for doses of 25, 50 and 100 mg/kg of the extract in the primary response ranged from 5.1±0.7 to 6.5±0.5 compared to the negative control (8.5±0.9). The corresponding data for secondary response was 4.6±0.7 to 6.6±0.5 compared to 10.8±0.7 in negative control.

Conclusions: Although, the extract showed the capacity to augment lymphocyte proliferation in the presence of mitogen or alloantigen, humoral antibody synthesis in both primary and secondary response was inhibited.

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Key Words • Plants, medicinal • lymphocyte proliferation • antibody production

Introduction

Echium amoenum, is a herbal plant belonging to the *Boraginaceae* (ox-tongue) family and grow widely in the northern highlands of Iran.

The best known species of this family, *Borago officinalis* is indigenous (semi-arid regions) of western Europe and naturalized in the eastern United States. It is also cultivated for its delicate, blue flowers. The leaves of this plant are tongue like bristled while the seed contains a variety of fatty acids notably Y-linoleic acid, a prostaglandin precursor.

In western traditional medicine, crude leaves has been used as diuretic, demulcent, emollient and expectorant.

Echium amoenum, has not been reported to grow in Europe or other parts of the world and is restricted to the northern parts of Iran. In Iranian traditional medicine, flower heads of this herb has long been used as a tonic and tranquilizer. Avicenna, the renowned Persian scientist and physician of the middle age in his epochal work "Canon of Medicine" (980-1037 AD) recommended the ash as well as other preparations of the plant in the treatment of oral ulcers and inflammations. A

concoction of this herb mixed with wine was believed to bring about euphoria and when mixed with honey it was used to ease heart palpitations. Makhzan-Al-Adviya written by Khorasani, a historic Iranian physician, described *Echium amoenum* as being a remedy for cough, sore throat, pneumonia and dyspnea. It was also recommended in the treatment of a range of eruptive fevers of children.

Phytochemistry analyses of other species of *Echium* has revealed the presence of pyrrolizidine alkaloids, Echinone and Echinofuran¹⁻³. Some anti-microbial and anti-inflammatory effects of these species have been documented in western studies.⁴⁻⁵

Immunological mechanisms have been implicated in the pathogenesis of a number of above-mentioned illnesses. This study was designed to investigate whether modulation of immunological reaction might underlie the folkloric efficacy of *Echium amoenum*.

This study examined the effects of hydrochloric extract of *Echium amoenum* on:

- I) spontaneously mitogenic activity on lymphocytes
- II) in vitro lymphocyte proliferation and mixed lymphocyte reaction, and
- III) in vitro humoral antibody response

Materials and Methods

Preparation of the extract:

Samples of fresh flower heads of *Echium amoenum* were collected during June from Mazandaran province. Plant materials were washed, dried and then 120 g of shade-dried powder was extracted with 70% ethanol. The yield (w/w) of the extract was 33.3%.

Humoral antibody response:

Six groups of 5 mice were immunized intraperitoneally (ip) with 5x10⁹ SRBC on days 0 and +7. In four groups, different doses of *Echium* extract (1-100 mg/kg) was administrated on days -2, -1, 1 and 2 of immunization (ip). The mice in the fifth group were injected with levamisole as an immunopotentiating agent (2 mg/kg, ip) on the same day. The sixth group was considered as non-treated control and injected only with normal saline. Blood samples were obtained from each mouse on day +7 for primary response and on day +14 for secondary response. Antibody titer was determined by hemagglutination assay as described previously.⁶ To 25 µl of two-fold diluted serum samples in V-shape micro-titration plates, 25 µl of 0.1% SRBC suspension was added. After 1 hour of incubation the highest dilution of serum samples which caused hemagglutination was considered as antibody titer and the mean log₂ of the titers were determined.

Lymphocyte proliferation assay:

Peripheral blood lymphocytes (PBL) from healthy individuals were separated using Ficoll-hypaque (Biotest, Germany) gradient centrifugation. Thymocytes prepared by teasing the thymus obtained from patients undergoing open-heart surgery. Appropriate concentrations of the cells were added to each well of a tissue culture plate (Nunc, Denmark). Then the same volume of media containing two-fold concentrations of 0.1 to 800 µg/ml of the extract was added. Two series of the cells were cultured without the extract and with 20 µg/ml of phytohemagglutinin (PHA) (Bahar-Afshan Co, Iran) for PBL cells, and 10 µg/ml of Concanavalin A (Pharmacia, Sweden) for thymocytes. The effect of the extracts also on lymphocyte proliferation in the presence of sub-optimal dose of PHA was assessed. To each well of tissue culture plate containing the cells, PHA (10 µg/ml) and different concentrations of the extracts were added. After 3 days of incubation, [³H]-Thymidine (Amersham Int. Plc) (0.5 µci/well) was added and then harvesting was done. CPM was measured using a β-counter (Pharmacia, Sweden). The mean CPM value of each of the three independent experiments performed in triplicate, and then the

stimulation index (SI) was calculated.

Mixed lymphocyte reaction:

100 μ l of each single cell suspension of PBL from two unrelated individuals were mixed in each well of microtissue culture plate and co-cultured in the presence or absence of different concentrations of the extract in triplicate. After 5 days of incubation, the same procedure was performed as for lymphocyte proliferation assay.

Results

Hydroalcoholic extract of *Echium amoenum* was examined for its mitogenic activity on the human lymphocytes and thymocytes. As shown in Figure 1, the SI of all cultures treated with different concentrations of the extract was almost equal to zero, the same as negative control (SI < 0.07). Similarly, extract-treated thymocytes showed no significant differences with non-treated cells (SI < 0.06, data not shown). The results indicated that incubation of *Echium amoenum* extract in concentrations ranging from 0.1 to 800 μ g/ml had no direct stimulatory effect on the PBL and thymocyte proliferation. However, addition of 50 to 400 μ g/ml of the extract to the PHA-treated cultures significantly increased the lymphocyte proliferation (SI range: 1.29-1.44, p < 0.02). Thus, *Echium amoenum* was shown to have a co-stimulatory effect with PHA in lymphocyte proliferative response. In MLR, *Echium* extract with concentrations of 0.1 to 10 μ g/ml showed a significantly higher SI than non-treated cultures (SI range: 1.35-1.81, p < 0.01) indicating the stimulatory activity of *Echium amoenum* on lymphocyte response to allogenic cells. The highest proliferative activity was detected at 10 μ g/ml. At concentrations higher than 700 μ g/ml, both MLR and lymphocyte proliferation assay were inhibited.

The effect of the extract on humoral antibody response was also examined. As shown in Table 1, a dose-related decrease in humoral antibody titer due to the extract doses of 1 to 100 mg/kg is observed in treated mice both in primary and secondary response (p < 0.05). As the concentration of the injected extract decreased, the antibody titer increased to reach near the level of non-treated mice. The mean antibody titer log₂ for levamisole as the positive control was significantly higher than for non-treated mice (p < 0.03).

Discussion

Study of the possible immunostimulatory effects of herbal plants on cell-mediated immunity (CMI) is a matter of interest for many investigators. In several distinct studies, the capacity of herbal plants on lymphocytic proliferation in the presence of mitogens, allogenic cells, and the specific antigens has been studied.⁷⁻¹² In this regard, the immunostimulatory activity of garlic on CMI has been reported.⁸ Green tea has been proven to increase humoral and cellular activity.⁹ Ginseng enhances production of macrophages, B and T cells.⁹ *Echinacea* is being tried as an agent for immune stimulation¹⁰ and *Acanthospermum hispidum*, a tropical plant, has been shown to enhance the proliferation of T lymphocytes after stimulation with Con A or allogenic stimulator cells in the mixed lymphocyte culture.¹¹ The present study has focused on the influence of *Echium amoenum* extract on mitogen and alloantigen-induced lymphocyte proliferation. This herb has a high application in Iranian traditional medicine for the treatment of infections and inflammatory diseases. Whether the therapeutic efficiency of this herb may, in part, be mediated via its influence on the immune response is not known. No immunomodulatory study has been reported for either *Echium amoenum* or other *Echium* species. The results obtained in this study indicated that *Echium amoenum* has the capacity of increasing the cellular immune response. The extract showed no spontaneous stimulatory activity on the human lymphocytes

and thymocytes but when the cells were cultured with the extract in the presence of a suboptimal dose of PHA, a strong enhancement in the cell proliferation was observed. Similarly assessment of the effect of the extract on allogenic reaction, another indicative of CMI determined by MLR, showed that *Echium* is able to increase this response. A possible explanation for these effects might be the presence of some components similar to lectins in the extract which can bridge cells by binding to surface proteins on stimulated cells and may co-stimulate and facilitate cellular interactions.¹² The lymphocyte proliferation activity may also be due to the direct effect of *Echium amoenum* or may be mediated through activated release of cytokines such as IL-1, IL-2 and IFN-Y. In several studies, increase in cytokine production due to herbal plants has been shown,¹³⁻¹⁴ large quantities of IL-10 have been secreted in culture of non-activated peripheral blood mononuclear cells and allogenic cells with whole *Nigella sativa* proteins¹³ Seed extracts of *Aegineta L* induced IL-2, IFN-Y, TNF and IL-6 production and lymphocyte proliferation in vitro.¹⁴ These reports highlight the need for further study, in particular to investigate the possible action of *Echium amoenum* extract and its components in interfering with cytokine production.

Despite the stimulatory effects on cellular immunity, *Echium amoenum* showed an inhibitory effect on humoral antibody response. Alterations in antibody synthesis have been reported for other herbal plants.¹⁵⁻²⁰ In this regard, administration of heteropolysaccharides from the camomile flower to rats resulted in stimulation of development of the immune response to SRBCs.¹⁶ The aqueous extract of *Achillea talagonica* has shown a significant decrease in anti-SRBC hemagglutination titer in primary response,¹² and in another study administration of *Picrorhiza kurroa* in mice prior to immunization with SRBC resulted in a significant increase in antibody titer and plaque forming assay.¹⁸ The antibody production to T dependent antigen SRBC requires the cooperation of T and B lymphocytes.²¹ The inhibition of humoral response to SRBC could be due to extract-induced inhibited phagocytic functions of macrophages, the cells involved in antigen processing and presentation, or may be influenced by relative amounts of different cytokines produced at the site of T cell and B cell stimulation.²²⁻²⁴ It should be noted that different dosage schedules, timings and routes of administration of the antigen and the extract may influence different types of antibody response. In our study, the immunosuppression of humoral response was observed when the mice were treated intraperitoneally 1 and 2 days before and after injection of the SRBC. It is possible that on prolonged treatment, this inhibitory effect would be altered.

In conclusion, this study showed that anti-inflammatory and anti-infection properties of *Echium amoenum* are associated with stimulation of cellular immune response and suppression of humoral antibody synthesis. The stimulatory effect could indicate it's therapeutic potential for treatment of infections and the inhibitory action could partly be considered for anti-inflammatory effect of this herb. It is important to elucidate whether these distinct capabilities of *Echium amoenum* exerted by one component of *Echium* or by different components. Additional studies to find the relationship between these effects and the therapeutic usefulness of the herb accompanied with identification of active ingredients are in progress.

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Combination Chemotherapy and IL-15 Administration Induce Permanent Tumor Regression in a Mouse Lung Tumor Model: NK and T Cell-Mediated Effects Antagonized by B Cells¹

Andrei I. Chapoval,² Jane A. Fuller, Sergey G. Kremlev, Sonya J. Kamdar, and Robert Evans

Previous studies have demonstrated that IL-15 administration after cyclophosphamide (CY) injection of C57BL/6J mice bearing the i.m. 76-9 rhabdomyosarcoma resulted in a significant prolongation of life. In the present study, we investigated the immune response against the 76-9 experimental lung metastases after CY + IL-15 therapy. Administration of CY + IL-15, but not IL-15 alone, induced prolongation of life and cures in 32% of mice bearing established experimental pulmonary metastases of 76-9 tumor. The CY + IL-15 therapy resulted in increased levels of NK1.1⁺/LGL-1⁺ cells, and CD8⁺/CD44⁺ T cells in PBL. In vitro cytotoxic assay of PBL indicated the induction of lymphokine-activated killer cell activity, but no evident tumor-specific class I-restricted lytic activity. Survival studies showed that the presence of NK and T lymphocytes is necessary for successful CY + IL-15 therapy. Experiments using knockout mice implied that either $\alpha\beta$ or $\gamma\delta$ T cells were required for an antitumor effect induced by CY + IL-15 therapy. However, mice lacking in both $\alpha\beta$ and $\gamma\delta$ T cells failed to respond to combination therapy. Cured B6 and $\alpha\beta$ or $\gamma\delta$ T cell-deficient mice were immune to rechallenge with 76-9, but not B16LM tumor. B cell-deficient mice showed a significant improvement in the survival rate both after CY and combination CY + IL-15 therapy compared with normal B6 mice. Overall, the data suggest that the interaction of NK cells with tumor-specific $\alpha\beta$ or $\gamma\delta$ T lymphocytes is necessary for successful therapy, while B cells appear to suppress the antitumor effects of CY + IL-15 therapy. *The Journal of Immunology*, 1998, 161: 6977-6984.

Interleukin-15, a 14- to 18-kDa cytokine, has biological activities similar to those of IL-2 (1, 2). IL-15 has been shown to stimulate the growth of NK cells (3), activated peripheral blood T lymphocytes (4), $\gamma\delta$ T cells (5), and B cells (6). It has been reported recently that IL-15 induces the production of proinflammatory cytokines from macrophages (M ϕ)³ (7) and activates human neutrophils (8). Reports that IL-15 induces the expression of mRNA for perforin and granzymes in murine lymphocytes (9), activates human PBL for perforin-mediated lysis of melanoma and lung cancer tumor cells (10, 11), and induces the generation of CTL (1) and the maturation/differentiation of cytotoxic NK cells (12, 13) suggest that this cytokine may play an important role in antitumor immunity. Indeed, it was shown that administration of IL-15 prolonged survival of lymphoma-bearing mice (14) and suppressed pulmonary metastases induced by i.v. injection of sarcoma cells (15).

It was shown previously in this laboratory that IL-15 acted as an adjuvant when administered in combination with CY, significantly prolonging the life of mice bearing the i.m. implanted 76-9 rhabdomyosarcoma (16). Combination therapy was seen to induce an

increase in NK cells in vivo. These were shown to be cytotoxic in vitro against YAC-1 cells, and to exert antitumor effects when adoptively transferred to CY-treated tumor-bearing (TB) mice. Their lack of cytotoxic activity in vitro against the 76-9 tumor, together with little or no evidence for IL-15-induced MHC class I-restricted lysis, suggested that NK cell involvement in antitumor activity was probably indirect and mediated via its secretory products. However, the mechanisms of the combined action of CY and IL-15 on tumors still need to be clarified. It is established that CY augments delayed-type sensitivity reactions by eliminating suppressor T cells (17) or by increasing the production of Th1-related cytokines (18). It has been reported that CY increases the localization of effector cells in the tumor mass (19), to augment the antitumor action of adoptively transferred tumor-infiltrating lymphocytes in clinical trials (20), and increases therapeutic efficacy of IL-2 (21). In addition, as was shown in this laboratory, CY injection resulted in an increase in tumor-associated M ϕ , as well as NK cell and granulocyte precursors (22, 23). Thus, because of its reported ability to react with NK cells, M ϕ , granulocytes, T cells, and B cells, as cited above, it seems plausible to suggest that antitumor adjuvant activity of IL-15 may be mediated by activation of any or all of these cellular compartments following CY chemotherapy.

In this study, we examined the impact of IL-15 as an adjuvant to cancer chemotherapy using CY in an experimental pulmonary metastasis model. In addition, we explored the cellular compartments most likely to be involved in successful CY + IL-15 therapy.

Materials and Methods

Mice

Male C57BL/6J, C57BL/6J-Lyst^{b6} (B6.beige), C57BL/6J-Prkdc^{-/-}/S2J (B6.scid), C57BL/6J-Hfl11^{nude} (B6.nude), C57BL/6J-TCR β ^{lmm1mcm}

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³ Abbreviations used in this paper: M ϕ , macrophage; CY, cyclophosphamide; LAK, lymphokine-activated killer; TB, tumor-bearing.

(B6.TCR- $\beta^{-/-}$), C57BL/6-TCRd^{lmlMam} (B6.TCR- $\delta^{-/-}$), C57BL/6-TCRb^{lmlMam}-TCRd^{lmlMam} (B6.TCR- $\beta\delta^{-/-}$), and C57BL/6-Igh-6^{lmlCgn} (B6.IgH-6^{-/-}) mice 8–10 wk old were obtained from The Jackson Laboratory Animal Resources Unit (Bar Harbor, ME). The absence of T cells in the TCR knockout and nude mice, and B cells in the B cell-deficient mice was confirmed by flow cytometry analyses. The absence of cytotoxic NK cells in beige mice was confirmed in cytotoxicity assays against YAC-1 cells.

Tumor cells

76-9 tumor is a syngeneic B6 3-methylcholanthrene-induced, weakly immunogenic rhabdomyosarcoma described previously (24). The tumor was passed *in vivo* in B6 mice every 2–3 wk. Tumor cell suspensions were prepared from solid tumor, as previously described (25). Briefly, i.m. tumor nodules were first mechanically dissociated into 2–4-mm fragments, and then enzymatically digested at 37°C for 1 h in RPMI 1640 containing 1 µg/ml deoxyribonuclease I (Sigma, St. Louis, MO), 250 µg/ml collagenase (Sigma), and 250 µg/ml papain (Sigma). The resulting tumor cell suspensions were washed, resuspended at desired concentrations, and used for i.v. injection into mice.

Preparation of PBL

PBL were obtained by modification of the methods described previously (26). In brief, blood was collected from the tail vein, diluted immediately in serum-free RPMI 1640 containing 50 mM EDTA, and washed by centrifugation for 10 min at 170–190 × g. The pellet was lysed using ice-cold lysing buffer (154 mM NH₄Cl, 1.5 mM KHCO₃, 0.1 mM EDTA, pH 7.2) for 5 min. After centrifugation, cells were washed three times to remove debris that contained red cell ghosts and residual platelets that sedimented above the cell pellet. The remaining white cells were suspended in medium, as required, and used in experiments.

In vivo treatment studies

Preliminary experiments indicated that injection of B6 mice with 10⁵ 76-9 tumor cells was a minimal dose that resulted in the development of pulmonary metastases that could not be cured with CY alone, but were sensitive to therapy with CY + IL-15. Pulmonary metastases developed after injection of 5 × 10⁴ or fewer 76-9 cells were curable with CY alone. Thus, in further experiments, we used 10⁵ 76-9 tumor cells as a minimal dose for development of pulmonary metastases not sensitive to chemotherapy alone. On day 0, mice were injected i.v. into the tail vein with 76-9 tumor cells (5 × 10⁵) to establish pulmonary tumors. Ten days later, mice were treated i.p. with single dose of 200 mg/kg body weight of CY (Cytotoxan; Bristol Myers Squibb, Princeton, NJ). Human rIL-15 (sp. act. of 4.45 × 10⁷ U/mg; Immunex, Seattle, WA) was given by i.p. injection for 20 days at a dose of 10 µg/mouse/day starting 24 h after CY treatment. Survival of TB mice was monitored every day. Mice that became moribund due to lung tumors (usually between 35 and 40 days after tumor inoculation for TB mice treated with CY alone) were killed for humane reasons. Mice surviving longer than 120 days posttumor injection were considered as cured. During the course of therapy, mice were bled (200 µl of blood from mouse) at various time points. PBL were isolated from the combined blood samples and used in cytotoxicity assays and flow cytometry analysis. In one experiment, randomly selected TB mice treated with CY ± IL-15 were killed at day 35 after 76-9 tumor inoculation, and lungs were infused with a 15% solution of India ink and bleached by Fekete's solution (27).

Flow cytometry

Biotin-conjugated anti-LGL-1 (clone 4D-11; The Jackson Laboratory), FITC-labeled anti-CD8 (clone 53-6.72; The Jackson Laboratory), phycoerythrin-labeled anti-CD44 (clone IM7.8.1; PharMingen, Los Angeles, CA), and phycoerythrin-labeled anti-NK1.1 (PK136; PharMingen) mAb were used to analyze the phenotype of PBL isolated from normal or TB mice treated with CY + IL-15. For that PBL were incubated at 4°C for 30 min with mAb, washed in PBS containing 5% FBS. Cells treated with biotin-conjugated mAb were cultured for additional 30 min at 4°C with FITC-labeled streptavidin and washed in PBS. Stained cells were analyzed using the Becton Dickinson FACScan.

Cytotoxicity assay

Cytotoxicity of PBL was measured in a standard 4-h ⁵¹Cr release assay. The tumor cell targets used were YAC-1 (NK cell sensitive), 76-9 rhabdomyosarcoma (H-2^b), C26 colon carcinoma (H-2^d), and B16LM melanoma (H-2^b). All target cells were maintained *in vitro* in RPMI 1640 medium (BioWhittaker, Walkersville, MD) supplemented with 2-ME (5 × 10⁻⁶ M; Sigma), gentamicin (50 µg/ml; Sigma), and 10% heat-inactivated

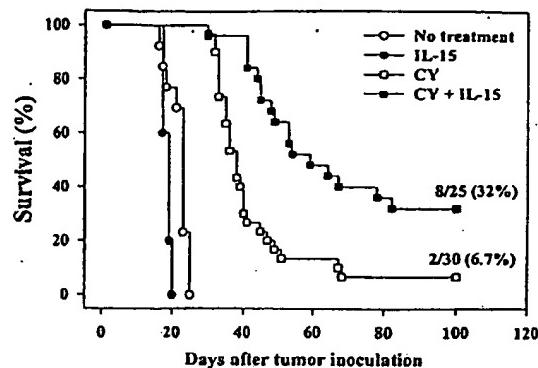


FIGURE 1. Survival of mice bearing established experimental pulmonary 76-9 rhabdomyosarcoma metastases. B6 mice were injected i.v. with 5 × 10⁵ of 76-9 tumor cells. Ten days later, mice were injected with 200 mg/kg CY (□), followed 24 h later by 20 daily injections of IL-15 (10 µg/injection (○)). Control tumor bearers were injected daily with vehicle (○) or IL-15 (●). The data represent the mean values of five independent experiments.

FBS (Atlanta Biologicals, Norcross, GA). Effector PBL and ⁵¹Cr-labeled target cells (4 × 10³ cells/well) were combined in 96-well V-bottom plates (Rainin, Wodurn, MA) at various E:T ratios and incubated for 4 h at 37°C and 5% CO₂; 100 µl/well of supernatant was then withdrawn, and radioactivity was measured in a gamma counter (Wallac, Gaithersburg, MD). Spontaneous release of ⁵¹Cr (incubation of target cells with media alone) was less than 15% of maximum release (incubation of target cells with 5% SDS detergent). There were three replicates for each sample. Data were expressed as percentage of cytotoxicity calculated from the following formula: % cytotoxicity = (test cpm – spontaneous cpm)/(maximum cpm – spontaneous cpm) × 100.

Statistics

All data were analyzed by using the Student's *t* test (SigmaPlot), or χ^2 test for survival studies, whereby $p < 0.05$ indicated that the value of the test sample was significantly different from that of the relevant controls.

Results

IL-15 administration after CY injection prolongs the life and cures TB mice

Lung metastases were established in B6 mice, as described. Ten days later, TB mice received an i.p. injection of CY (200 mg/kg) and daily i.p. injections of IL-15 (×20 at 10 µg/injection) beginning 24 h after CY. The data in Fig. 1 summarize five independent experiments and show that 32% (8 of 25) of CY + IL-15 mice were cured, the remaining mice showing significant prolongation of life compared with mice receiving CY treatment alone, in which 6.7% (2 of 30) were cured. The difference between groups of mice treated with CY alone or CY + IL-15 was statistically significant ($p < 0.005$), as calculated at 100 days after tumor inoculation. Fig. 2 shows that by day 35 after tumor inoculation, there were no visible tumor nodules in the lungs of mice receiving CY + IL-15 therapy in contrast to lungs from CY-treated controls. Mice that were cured by either CY alone or combined CY + IL-15 therapy were resistant to a subsequent i.m. challenge with 10⁴ 76-9 tumor cells, while challenge with the irrelevant syngeneic B16LM tumor resulted in tumor growth (data not shown), indicating the presence of immunologic memory.

IL-15 induces an increase in the number of NK1.1⁺/LGL-1⁺ cells and CD8⁺/CD44⁺ cells

PBL from TB mice that had been treated with CY and 20 daily injections of IL-15 (10 µg/day) were analyzed by flow cytometry

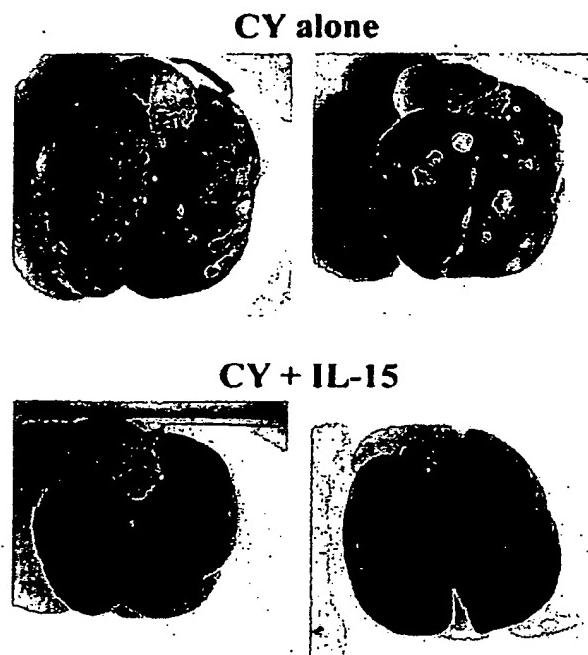


FIGURE 2. Effect of CY + IL-15 therapy on the number and size of established experimental pulmonary metastases of 76-9 tumor. Mice were treated as in Fig. 1. Thirty-five days after tumor inoculation (=25 days after CY injection), lungs from randomly selected mice treated with CY alone (upper panel) or CY + IL-15 therapy (lower panel) were removed and perfused with India ink. The presence of tumor lesions in two lungs in each group is shown.

for the expression of multiple Ags, including NK1.1, LGL-1, CD4, CD8, CD44, B220, Gr-1, MAC-1, and F4/80 as markers of the major types of potential effector cells. As was shown previously, injection of CY alone decreased the absolute number of PBL (22). Multiple injections of IL-15 into CY-treated TB mice did not significantly change the absolute number of PBL, but increased the proportions of NK1.1, LGL-1, CD8, and CD44 cells. The data presented in Fig. 3 is a typical dot plot of PBL isolated from TB mice injected with CY or CY + IL-15. Cells were double stained for the expression of NK1.1 and LGL-1 (upper panel) or CD8 and CD44 (lower panel). It is seen that 20 daily injections of IL-15 induced increase in NK1.1⁺/LGL-1⁻ cells (sixfold) and NK1.1⁺/LGL-1⁺ cells (17-fold). The percentage of CD8⁺/CD44⁺ cells in PBL from CY + IL-15-treated mice was also five times higher than in control mice (injected with CY alone), while the percentage of CD8⁺/CD44⁻ was the same in both groups of mice. The above changes in NK1.1, LGL-1, CD8, and CD44 expression were also seen in non-TB mice after injection with CY + IL-15, indicating that this was not related to the presence of tumor and depended on IL-15 administration (data not shown). Administration of IL-15 into normal or TB mice that did not receive CY treatment resulted in lower levels of NK1.1⁺/LGL-1⁺ and higher levels of CD8⁺/CD44⁺ cells compared with mice treated with CY + IL-15 (data not shown). The changes in the expression of the other Ags relative to the appropriate controls were not significant and are not shown.

It was evident the maximum accumulation of NK and CD8⁺/CD44⁺ T cells in peripheral blood was dependent on the number of IL-15 injections after CY injection. This is shown in Fig. 4. TB mice were injected i.p. with CY, followed 24 h later by daily injections of 10 µg of IL-15. PBL (from a pool of five mice per

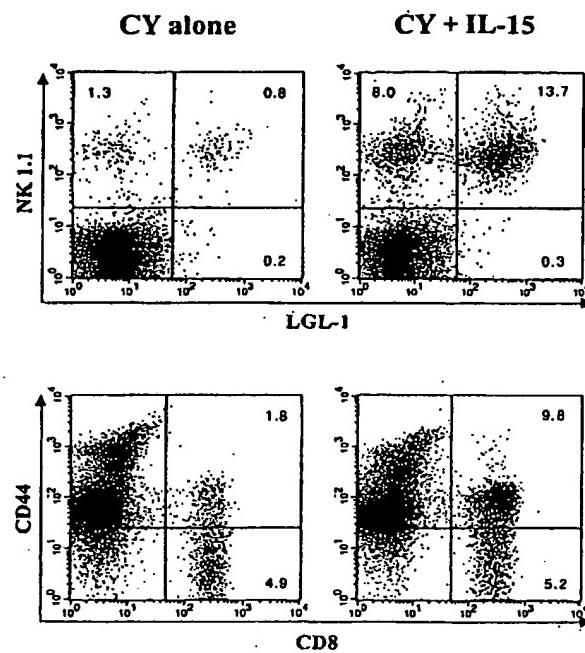


FIGURE 3. Flow cytometry analysis of PBL from B6 mice with pulmonary metastases of 76-9 tumor receiving either CY treatment or combination CY + IL-15 therapy. Cells were isolated 24 h after 20 daily injections of IL-15 (right panel) or vehicle (left panel) and analyzed for NK1.1, LGL-1, CD8, and CD44 Ag expression. The percentages are shown in each quadrant. The representative of more than 15 experiments is shown.

group) were collected 24 h after 5, 10, 15, and 20 injections of IL-15. As shown, the number of NK cells in PBL reached the maximum level after 10 injections of IL-15 and declined slightly after 15 injections. By day 7 after the twentieth injection of IL-15,

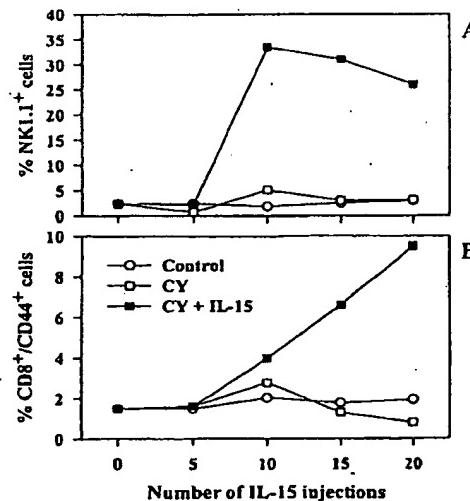


FIGURE 4. The effect of multiple injections of IL-15 on the percentage of circulating NK1.1⁺ and CD8⁺/CD44⁺ cells. PBL were isolated from B6 mice with pulmonary metastases of 76-9 tumor treated with CY (□) or combination CY + IL-15 therapy (■) at the times shown. Cells isolated from normal B6 mice (○) were also analyzed as a baseline control. The staining procedures were as described for Fig. 3.

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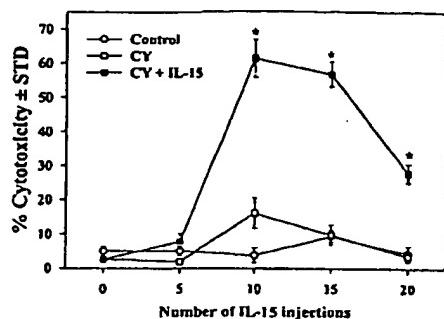


FIGURE 5. NK-mediated cytotoxicity of PBL isolated as described for Fig. 4. PBL were incubated with NK cell-sensitive YAC-1 in a 4-h ^{51}Cr release assay. Values represent the mean \pm SD of triplicate wells at an E:T cell ratio of 50:1. *, $p < 0.05$ compared with TB mice treated with CY alone.

the percentage of NK cells returned to the level seen in control normal B6 mice (data not shown). In contrast, the percentage of CD8 $^+$ /CD44 $^+$ cells increased in parallel with the number of IL-15 injections (Fig. 4B) and remained high for at least 21 days after the last injection of IL-15 (data not shown).

IL-15 induces LAK cell, but not tumor-specific, class I-restricted activity in vivo

To determine whether the increased levels of IL-15-induced NK1.1 $^+$ and CD8 $^+$ cells were associated with increased cytotoxicity, PBL isolated as above were also tested for cytotoxicity in a standard 4-h ^{51}Cr release assay. Fig. 5 shows that PBL isolated from TB mice treated with CY and IL-15 were highly cytotoxic against NK cell-sensitive targets (YAC-1). Similar to the accumulation of NK1.1 $^+$ cells in PBL, the peak of NK-mediated cytotoxic activity occurred by 10–15 injections of IL-15 and declined thereafter. Cytotoxicity above background levels was not detectable 7 days after the twentieth injection of IL-15 (data not shown).

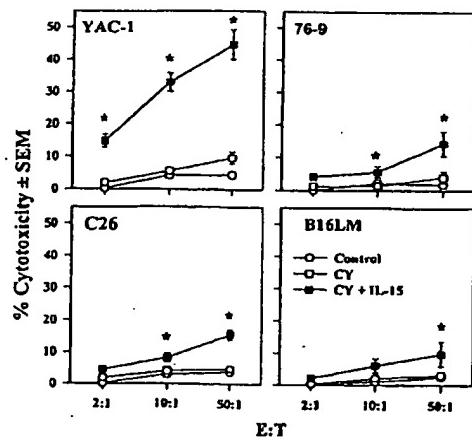


FIGURE 6. Cytotoxicity of PBL isolated from B6 mice with pulmonary metastases of 76-9 tumor treated with CY or CY \pm IL-15. PBL isolated 24 h after 10 injection of IL-15 were incubated with NK-sensitive YAC-1, the specific 76-9, the C26 (BALB/c), and B16LM (C57BL/6) targets in 4-h ^{51}Cr release assay. Values represent the mean \pm SEM of two to five independent experiments (pool of 5 mice per experiment). *, $p < 0.05$ compared with TB mice treated with CY alone.

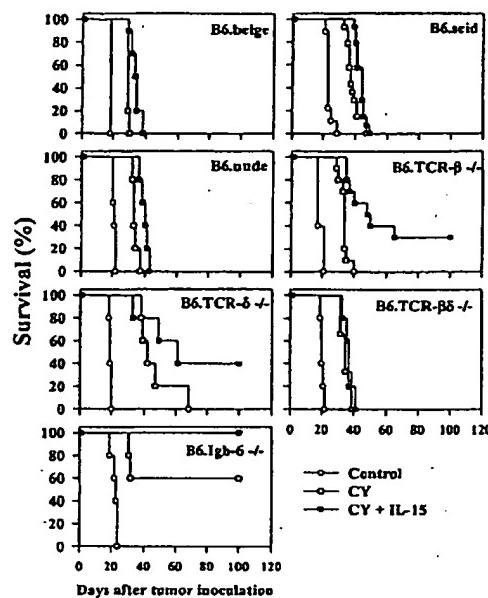


FIGURE 7. Survival of various mutant mice bearing established experimental pulmonary 76-9 tumor metastases. Mice were injected i.v. with 5×10^5 76-9 tumor cells. Ten days later, mice were injected i.p. with 200 mg/kg CY (□), vehicle (○), or daily injections of IL-15, as described in Fig. 1. The data represent the combined values of two independent experiments.

PBL isolated after 10 injections of IL-15 expressed high levels of cytotoxicity against YAC-1, and a lower level of lytic activity toward 76-9, C26, and B16LM target cells (Fig. 6), suggesting NK and LAK cells activity. The cumulative data from eight independent experiments indicated wide variability in 76-9 cell lysis (e.g., in one experiment, lysis was 40–50%; in four experiments, lysis was 10–30%; and in three experiments, lysis was 0–10%). Variability was also seen in the lysis of BALB/c C26 colon carcinoma target cells (0–20% lysis) and B6 B16LM melanoma cells (0–30%). The increased cytotoxicity against syngeneic B16LM melanoma cells suggested the presence of LAK cells, but not Ag-specific cytolytic activity. In addition, no significant increase in tumor sp. act. in PBL from CY + IL-15-treated TB mice was seen in tumor growth-inhibition assays, in which effector cells were cultured with target cells (YAC-1, 76-9, C26, B16LM) for 96 h.

Cellular requirements in vivo for successful CY + IL-15 therapy

To determine whether NK, T, or B cells were responsible for the antitumor action of CY + IL-15 therapy, survival studies were conducted using B6 mice with impaired NK cell activity (B6.beige), T and B cell deficient (B6.scid), T cell deficient (B6.nude), lacking of B cells (B6.IgH-6), and induced mutants deficient in $\alpha\beta$ T cells (B6.TCR- $\beta^{-/-}$) or $\gamma\delta$ T cells (B6.TCR- $\delta^{-/-}$) or both $\alpha\beta$ and $\gamma\delta$ T cells (TCR- $\beta\delta^{-/-}$). Mice were inoculated i.v. with 5×10^5 76-9 tumor cells. Ten days later, they were injected with CY (200 mg/kg), followed 24 h later by 20 daily injections of IL-15. Fig. 7 summarizes the survival data. It is seen that IL-15 in combination with CY did not improve the survival rate in B6.beige, B6.nude, or B6.scid mice, but resulted in cures in 30% of the B6.TCR- $\beta^{-/-}$ mice and in 40% of the B6.TCR- $\delta^{-/-}$ mice. In the double knockouts B6.TCR- $\beta\delta^{-/-}$, therapy with CY + IL-15 had no effect on survival compared with treatment with CY alone. The most effective CY + IL-15 therapeutic effect was seen

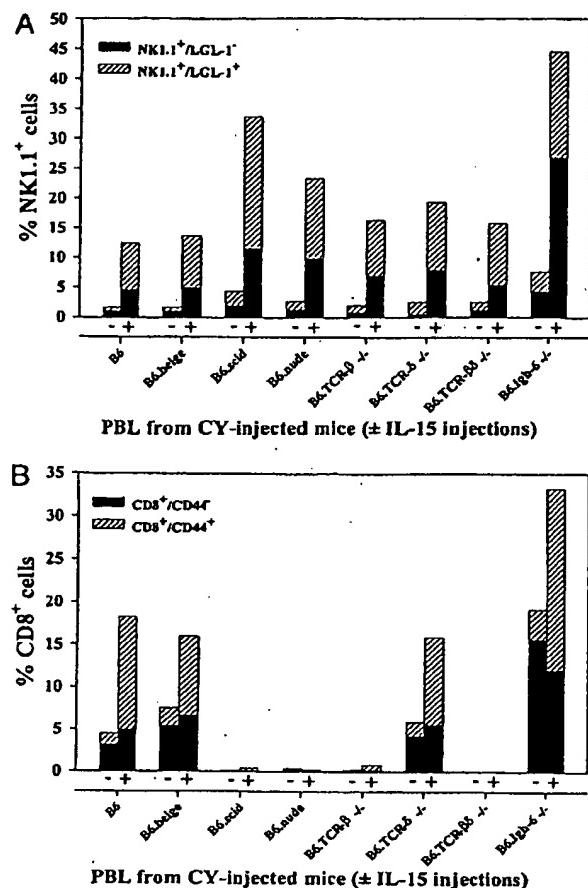


FIGURE 8. Percentage of NK1.1⁺, LGL-1⁺ (A), and CD8⁺/CD44⁺ (B) cells in PBL isolated from B6 and mutant mice bearing pulmonary metastases of 76-9 tumor treated with CY or CY + IL-15 therapy. Cells were isolated from CY-treated mice 24 h after 20 daily injections of vehicle or IL-15 and analyzed by flow cytometry for the expression of the four Ags. Baseline controls are represented by PBL from untreated normal B6 mice. The representative of five independent experiments is shown.

in the B6.IgH-6^{-/-} B cell-deficient mice, in which 100% of the mice were cured, suggesting a suppressor role for B cells toward CY + IL-15 therapy. Even in the CY control group, 60% of the mice were cured. Those mice deficient in $\alpha\beta$ T cells, $\gamma\delta$ T cells, or B cells surviving longer than 120 days were resistant to a challenge with 10^4 76-9 tumor cells, but not with B16LM tumor cells (data not shown). Since the cells other than NK cells may be defective in B6.beige mice, an attempt was made to deplete NK cells in vivo by administration of NK1.1 Ab to B6 mice before and after injection of tumor cells and the administration of combination CY + IL-15 therapy. Unfortunately, although depletion of circulating and splenic NK cells was successful, the administration of IL-15 resulted in the reappearance of peripheral NK cells. As reported by Puzanov et al. (12), IL-15 induces maturation and proliferation of bone marrow-associated NK cell precursors.

To determine whether parallel increases occurred in NK1.1⁺/LGL-1⁺ and CD8⁺/CD44⁺ cells as well as cytotoxicity, as shown for B6 mice (see Figs. 4 and 5), following CY + IL-15 therapy, PBL were isolated from these mutant mice after 20 injections of IL-15. The data in Fig. 8A show that IL-15 administration induced a variable increase in both NK1.1⁺/LGL-1⁺ and NK1.1⁺/LGL-1⁺

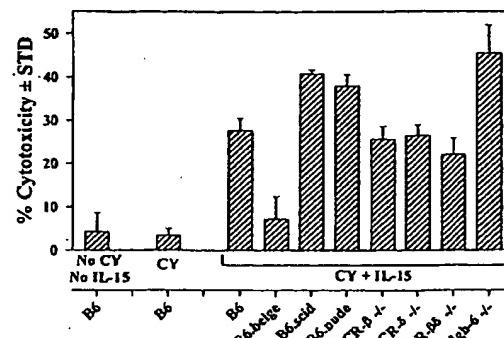


FIGURE 9. Cytotoxicity of PBL isolated from B6 and mutant mice with pulmonary metastases of 76-9 tumor, treated with CY or CY + IL-15 therapy. Cells were isolated from CY-treated mice 24 h after 20 daily injections of vehicle or IL-15 and incubated with ⁵¹Cr-labeled YAC-1 target cells, as described for Fig. 5. Values represent the mean \pm SD of triplicate wells at an E:T cell ratio of 50:1.

cell populations in all strains, including beige. The high levels of NK1.1⁺ cells in B6.scid and B6.IgH-6^{-/-} B cell-deficient mice can probably be explained by the absence of the major population of B cells, which account for up to 70% of the total PBL in B6 mice. This explanation serves to explain the increase in CD8⁺/CD44⁺ cells in B cell-deficient mice (Fig. 8B). The absence of CD8⁺ cells in PBL from B6.scid, B6.nude, and B6.TCR- β ^{-/-} mice confirms the T cell deficiency in these mice. Fig. 9 summarizes the NK cytotoxicity data. An increase in cytotoxic PBL (versus YAC-1 cells) was seen in all mutant mice, relative to background levels seen in untreated B6 mice or CY-treated TB mice, with the exception of B6.beige PBL, which expressed background levels only. PBL from B cell-deficient mice treated with CY and IL-15 show the highest cytotoxic activity, presumably because of the absence of B cells that increased the proportion of NK cells. All PBL that showed cytotoxicity against YAC-1 cells were also able to lyse 76-9, C26, and B16LM targets, but significant variation in killing of these targets was seen, as described above.

Discussion

The current data indicate that treatment of mice bearing established experimental 76-9 rhabdomyosarcoma pulmonary metastases with CY + IL-15 induced cures in 32% of mice, while only 6.7% of mice were cured with CY alone. Since the complete eradication of experimental pulmonary metastases could be achieved only by treatment with the combination of CY and IL-15, but not with either agent alone, the data imply that IL-15 potentiated the antitumor action of CY. First, the oncolytic action of CY may result in a smaller tumor mass that may be more amenable to rejection by host-mediated immune responses. While CY induces a reduction in the size of the 76-9 lung tumors, it is unlikely that tumor size alone determines whether IL-15 induces antitumor activity, since tumors developing from smaller tumor inocula were not more sensitive to IL-15 administration. Second, it has been reported that CY injection inhibits suppressor cell activity associated with spleen cells (17). In addition, it was shown previously that spleen cells from 76-9 TB mice could be used as a potent source of sensitized T cells that were therapeutically active when adoptively transferred to CY-injected TB mice (28). Other reports similarly suggest that in some tumor models, suppressor cells are absent or exert minimal effects in CY-injected mice (29). Third, it

has been shown in the 76-9 tumor model that CY injection was followed by an increase in the expression of Th1-related cytokine genes such as IFN- γ , IL-2, and TNF- α at the tumor site (24, 30). Since IL-15 has been reported to induce the production of TNF- α and IFN- γ from T and NK cells (2, 31, 32), its administration after CY injection may further promote the production of Th1-related cytokines. This in turn may augment T cell immune reactions at the tumor site, including the generation and activation of CTL and LAK cells. Finally, it has been reported that CY injection resulted in an increase in M ϕ , NK cells, and polymorphonuclear precursor at the tumor site (22, 23). In view of the reports that IL-15 may activate each of these cell types (7, 8, 12, 13), the administration of IL-15 in combination with CY therapy clearly has the potential to accentuate the antitumor roles that each or all of these cells express.

The flow cytometry data indicated that when TB mice received combination CY + IL-15 therapy, there was a substantial increase in the proportions of NK and CD8 $^{+}$ T lymphocytes. Increases in peripheral blood CD4 $^{+}$ T lymphocytes, B cells, M ϕ , or granulocytes were not seen. The question raised was whether the increased levels of NK cells or CD8 $^{+}$ cells, or both, were responsible for the observed in vivo antitumor effects. Although high cytotoxic PBL activity was generated toward YAC-1 cells, only relatively low cell cytotoxic activity was generated against the 76-9 targets. Moreover, the specific tumor targets were no more susceptible to cytotoxic cells than the B16LM melanoma or C26 targets, suggesting LAK but not T cell cytotoxicity in PBL. In some experiments, the data suggested significantly higher cytotoxic activity toward 76-9 cells compared with the other two targets, but this was not reproducible over the full range of experiments. This low level of LAK cell activity observed in PBL was induced in the various natural and induced mutant mice and did not correlate with in vivo antitumor effects induced by CY + IL-15 therapy. Nevertheless, previous data indicated that NK1.1 $^{+}$ /LGL-1 $^{+}$ cells expanded in vitro with IL-15 expressed potent antitumor effects in vivo when adoptively transferred to CY-treated 76-9 TB mice (16). These expanded cells showed considerable NK cell activity in vitro, but only low LAK cell activity. Clearly, in vivo activity was not reflected by in vitro cytotoxicity data. Similarly, it seems unlikely that CD8 $^{+}$ /CD44 $^{+}$ T cells detected in PBL, putative memory cells (33) played a direct role in the antitumor effects generated by CY + IL-15 therapy since IL-15 administration induced an increase in non-TB mice. If within this population there is a tumor-specific subset of memory T cells, this was not evident based on the in vitro cytotoxicity data. However, the findings that those $\alpha\beta$ and $\gamma\delta$ T cell-deficient mice that were cured by CY + IL-15 therapy were shown to be resistant to a challenge with 76-9 cells, but not to the syngeneic B16LM melanoma cells, indicated that tumor-specific effectors had been generated. As discussed previously in the context of spleen cells (16), to what extent the in vitro activity of PBL reflects events occurring at the tumor site during the generation of antitumor activity remains to be elucidated.

In an attempt to determine what cells are required for successful CY + IL-15 therapy, the survival of mutant mice in response to combination therapy was evaluated. The overall data suggested that NK cells and T cells expressing either $\alpha\beta$ -TCR or $\gamma\delta$ -TCR were required for a positive antitumor effect, while B cells appeared to be antagonistic to positive antitumor responses. The evidence concerning NK cells based on the use of B6.beige mice is somewhat equivocal. First, unsuccessful therapy in B6.beige mice may be explained on the basis that other defective cells play important roles. For example, it has been reported that lysis mediated by cytolytic T cells is defective in B6.beige (34). Second, IL-15 administration resulted in increased numbers of NK cells and NK

cell-mediated cytotoxicity in B6.scid, B6.nude, and B6.TCR- $\beta\delta^{-/-}$ mice that failed to respond to CY + IL-15 therapy. This would indicate that if NK cells were required for antitumor activity, they did not appear to act independently of T cells and probably did not exert their effects toward 76-9 tumor cells by direct lytic activity. There is no question that the NK cells are activated, as measured by increased cytotoxicity and by expression of the activation marker B220 (16). Thus, as discussed previously (16), it seems more plausible to suggest that the involvement of activated NK cells in antitumor effects will be via their secretory products acting on other cell types, such as T cells or M ϕ . It is proposed that the therapeutic efficacy of IL-15-expanded NK cells adoptively transferred to CY-treated 76-9 TB mice is likely to be mediated by their secretory products orchestrating the generation of antitumor effectors.

On the other hand, the collective data from the experiments involving B6.scid, B6.nude, and TCR-deficient mice were compelling in that there was also an absolute requirement for T cells for successful CY + IL-15 therapy. The apparent alternative roles of $\alpha\beta$ and $\gamma\delta$ T cells in this regard are intriguing, since these two cell populations have different mechanisms of Ag recognition. It is well documented that $\alpha\beta$ T cells can kill tumor cells in an MHC class I-restricted manner (35). It also has been reported that $\gamma\delta$ T cells can lysis tumor target cells in an Ag-specific manner (36, 37). Reports that $\gamma\delta$ T cells may localize in the lung, as well as other epithelial tissues such as skin and intestine (38), suggest that $\gamma\delta$ T cells might be important in protecting the host against lung metastases. As cited above, IL-15 activates both $\alpha\beta$ and $\gamma\delta$ T cells (5, 39, 40). The findings that cured TB mice deficient in $\alpha\beta$ or $\gamma\delta$ T cells resisted a challenge with 76-9 cells, but not with the B16LM melanoma cells, indicated that tumor-specific effectors had been generated in vivo. As discussed above, the failure of the in vitro cytotoxicity assays to show the presence of tumor-specific T cells would suggest that cytolytic T cells are not generated systemically, but only at the tumor site.

The exciting finding that the most successful antitumor effects induced by CY + IL-15 therapy were seen in the TB B6.IgH-6 $^{-/-}$ mice deficient in B lymphocytes provides for the first time a likely pathway by which therapeutic efficacy is regulated. The role of B cells in antitumor immunity is rather controversial. In several mouse models and in melanoma patients, it has been reported that the clinical outcome of immunotherapy was associated with B cell immune responses (41, 42). In addition, it was shown that B cells play an essential role in host protection against virus-induced tumors (43). However, it is evident that depletion of B cells by Abs against mouse IgG or IgM enhanced rejection of allogeneic or chemically induced tumors (44, 45). Our current data indicate that the absence of B cells is associated with enhanced antitumor effects, suggesting that in replete B6 mice, the presence of B cells antagonizes antitumor effects. We can only speculate at this time as to the mechanism of action involved. It was shown that cell-mediated antitumor immunity can be blocked by Ab or Ab-Ag complexes (46, 47), and in the absence of B cells this inhibition did not occur. In view of the proposed dependence of successful CY + IL-15 therapy on NK cells and T cells, a more plausible candidate may be based on reports that B cell-deficient mice are unable to mount significant Th2 responses, while Th1 responses are reported to be enhanced (48, 49). Th2-related cytokines such as IL-4 and IL-10 were shown to suppress IL-15-induced activation of T lymphocytes and NK cells (31, 50). Thus, in the absence of B cells and suppressive Th2 factors, IL-15 may amplify Th1-dependent reactions, including the generation of antitumor cytotoxic effectors.

In conclusion, we have shown that the combined treatment of CY and IL-15 induced a significant incidence of permanent regression of experimental metastases of the 76-9 rhabdomyosarcoma. This was associated with an increase in activated peripheral blood NK cells and CD8⁺/CD44⁺ memory T cells. Successful therapy required the presence of either $\alpha\beta$ or $\gamma\delta$ T cells, and the absence of both subsets abrogated the therapeutic efficacy. Of considerable interest in the context of understanding how the therapy works was the finding that the most effective therapeutic benefit was seen in B cell-deficient mice, suggesting that B cells or their products antagonize potential antitumor effector function. While neither the positive effects of CY + IL-15 therapy nor the negative effects of B cells have yet to be fully elucidated, in future experiments we will test the hypothesis that NK cells mediate their effects by amplifying the effects of Th1 cells whose products activate effector $\alpha\beta$ or $\gamma\delta$ T cells. From a practical standpoint, the antagonistic effect of B cells would suggest that depletion of B cells may improve the clinical outcome of combination CY + IL-15 therapy.

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TEPOXALIN, A NOVEL IMMUNOMODULATORY COMPOUND, SYNERGIZES WITH CSA IN SUPPRESSION OF GRAFT-VERSUS- HOST REACTION AND ALLOGENEIC SKIN GRAFT REJECTION

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Tepoxalin, a dual 5-lipoxygenase and cyclooxygenase inhibitor with nonsteroidal antiinflammatory effects, has recently been shown to suppress NF- κ B transactivation and inhibit T cell proliferation via a mechanism very different from cyclosporine (CsA). In this report, we demonstrate that this novel immunosuppressive effect of tepoxalin is manifested in *in vivo* transplantation models. Tepoxalin suppressed murine spleen cell proliferation in a mixed lymphocyte reaction (MLR) with an IC₅₀ of 1.3 μ M. Coadministration of tepoxalin and CsA in MLR cultures showed an additive inhibitory effect. Oral administration of tepoxalin at 12 mg/kg/day to mice suppressed local graft-versus-host (GVH) responses by about 40% ($n=10$). Combination of tepoxalin and CsA at suboptimal doses synergized their immunosuppressive effects on GVH responses ($n=20$). In skin transplantation, the median survival time of allogeneic BALB/cByJ (H-2^a) mouse skin grafted onto C3H/HeJ (H-2^b) mice was 10.5 days ($n=8$), and was prolonged to 15.0 days ($n=8$) for recipient mice administered tepoxalin at 50 mg/kg/day. Co-administration of suboptimal doses of tepoxalin (12.5 mg/kg/day) and CsA (50 mg/kg/day) prolonged skin graft rejections dramatically (55% of the grafts survived for more than 40 days, $n=8$). Taken together, these results demonstrate that tepoxalin is a potent immunomodulatory compound that, when combined with CsA, provides synergistic immunosuppressive activity. The fact that tepoxalin and CsA act on different transcription factors, NF- κ B and NFAT respectively, might explain the synergistic suppressive effects when both compounds were used. Tepoxalin could be an important addition to the cohort of immunosuppressive therapies currently used in solid organ and bone marrow transplantations.

The immune response in transplantation, which results in graft rejection and graft-versus-host (GVH) response, is primarily triggered by T cells through recognition of alloantigens (1-4). Suppression of immune response could be achieved using agents interfering with T cell activation and effector functions. The use of cyclosporine (CsA) as an immunosuppressant in transplantation has been documented (5, 6). CsA inhibits T cell activation by inhibiting the nuclear translocation of the nuclear factor NFAT (7, 8). However, CsA has associated toxicities and side effects when used at

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* Abbreviations: CsA, cyclosporine; CO, cyclooxygenase; GVH, graft-versus-host; LO, 5-lipoxygenase; MLR, mixed lymphocyte reaction.

therapeutic doses (9). Compounds that suppress T cell-mediated immune response with mechanisms different from that of CsA will undoubtedly be valuable additions to the cohort of the current regimens.

Tepoxalin (5-(4-chlorophenyl)-N-hydroxy-[4-methoxyphenyl]-N-methyl-1-H-pyrazole-3-propanamide) was discovered originally as a dual inhibitor of 5-lipoxygenase (LO) and cyclooxygenase (CO) and exhibits potent nonsteroidal antiinflammatory activities in animal models of adjuvant arthritis (10-12). Recently we found that tepoxalin also inhibits OKT3-induced T cell proliferation via a mechanism very different from that of CsA (13). CsA is known to block IL-2 production after activation of T cells through TCR/CD3, whereas tepoxalin inhibits IL-2 induced signal transduction (13). An in-depth investigation of the mechanism of action reveals that tepoxalin inhibits predominantly NF- κ B activation (14), whereas CsA is most effective in blocking NFAT transactivation (7, 8). Because of these different mechanism of actions, a possible additive/synergistic effect of the combined tepoxalin and CsA treatment is expected. In this report, we demonstrate that tepoxalin is indeed effective in suppressing mixed lymphocyte reactions (MLR), GVH responses, and allogeneic skin graft rejections in mice. The synergistic effect of tepoxalin and CsA in immunosuppression was also studied. The possible mechanism of tepoxalin in immunosuppression and its potential clinical application are discussed.

MATERIALS AND METHODS

Mice. Inbred C57BL/6J, C3H/HeJ, and BALB/cByJ mice and B6D2F₁/J mice were obtained from the Jackson Laboratory (Bar Harbor, ME). Those used in experiments were male mice at about 6-10 weeks of age that weighed 18-25 g.

Preparation of test compounds. Tepoxalin, naproxen, and zileuton were synthesized by the R.W. Johnson Pharmaceutical Research Institute (Raritan, NJ). CsA (Sandimmuno L.V.) was from Sandoz (Quebec, Canada). For MLR experiments, stock solutions of tepoxalin, naproxen, and zileuton were prepared in DMSO at 30 mM and diluted to working concentrations in culture medium at the time of experiments. DMSO at concentrations equivalent to those of the test compounds were used as controls in MLR assays. For experiments of GVH responses and skin graft rejections, micritized tepoxalin and naproxen were suspended in 0.5% methylcellulose (Sigma, St. Louis, MO) at concentrations of 5 mg/ml or lower. The vehicle control was the equivalent volume of 0.5% methylcellulose. Zileuton was dissolved in 50% polyethylene glycol 200 (Sigma, St. Louis, MO), and the corresponding vehicle control was the equivalent volume of polyethylene glycol 200. CsA was diluted in saline. All compounds were dissolved in vehicle just prior to administration to mice at volumes of 0.01 ml per gram body weight.

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Lymphocyte proliferation assays. Single-cell suspensions from spleens were washed once with PBS and then resuspended in RPMI 1640 medium supplemented with 5% fetal bovine serum and 2-mercaptoethanol. Responder spleen cells (2.5×10^6) from C57BL/6J mice (H-2^b) were stimulated by 2.5×10^3 irradiated (2000 rads) spleen cells from B6D2F₁/J mice (H-2^{ab}). The responder and stimulator cells were cocultured in 250 μ l medium containing various concentrations of the tested compounds in the 96-well plates (bottom wells, Corning Inc., NY). After 5 days of stimulation, thymidine was added to the cultures (0.5 μ Ci per well) for 4 hr. Cells were harvested using a Tintec Harvester 96, MACH II (Tintec Inc., Orange, CT) and samples were counted using a Wallac Betaplate scintillation counter (Pharmacia, Uppsala, Sweden).

Cell viability test. Cell viability was assessed with the MTT assay. Spleen cells from C57BL/6J mice were prepared in RPMI 1640 medium supplemented with 5% fetal bovine serum and 50 μ M 2-mercaptoethanol. Spleen cells (2×10^6 /well) were stimulated with immunosuppressive anti-CD3 (Pharmingen) in the presence of tepezalin or its vehicle (DMSO) in 96-well culture plates (Corning Inc., NY). The MTT overtake assay was conducted by using the Celltiter 96 kit (Promega Corp.) and measured at 570 nm based on the conversion of a tetrazolium salt by viable cells and the resulting detectable blue formazone.

Graft-versus-host reactions. The GVH assay was based on the technique of Dorsch and Raser (15). Spleen cells from C57BL/6J mice were injected subcutaneously into the footpads of B6D2F₁/J mice. Seven hours later, the draining popliteal lymph nodes were removed, freed of fat and weighed. Mice injected in the footpads with saline had no reaction and were used as negative controls. Lymph nodes of these mice were also distinguishable from those injected with syngeneic spleen cells. Tepezalin was administered orally and CaA was given subcutaneously to mice daily starting one day before footpad injection unless otherwise specified.

Skin graft transplantation. C3H/HeJ mice (H-2^b) were anesthetized by intraperitoneal injection of 2.5% averin (0.016 ml/g body weight). A grafting bed (about 0.3 cm \times 1 cm) on the mouse tail was prepared by peeling off skin carefully to avoid bleeding. Tail skin of similar size was peeled from BALB/cByJ mice (H-2^a) and then placed onto the graft site in an opposite orientation according to the hair direction. The grafted skin was protected by a plastic tubing (inner diameter 0.5 cm, length 3 cm) held in place by wound clips for 5 days. Skin grafts were examined and scored daily. A graft was scored as rejected when more than 80% of the graft was necrotic. CaA was given subcutaneously to mice daily starting one day before skin transplantation until rejection of grafts. Tepezalin was given orally one day before transplantation and then daily starting one day after transplantation until graft rejection.

Data presentation and statistics. Data were analyzed using one-way analysis of variance and Dunnett's test. A parametric version was used if data were normally distributed as assessed by the Wilk-Shapiro test. Data which did not meet the assumptions of normality were tested using nonparametric version of the Dunnett's test.

RESULTS

Inhibition of MLR proliferations by tepezalin. We recently reported that tepezalin suppresses T cell proliferation and inhibits the activity of the transcription factor NF- κ B (13, 14). Long-term cell activation and proliferation are critical for the initiation of an antigen specific immune response. The transcription factor NF- κ B is also known to be involved in regulating the expression of many target genes in an immune response of polymers (17). The possible immunosuppressive effect of tepezalin is therefore studied. To determine whether tepezalin is capable of inhibiting the immune response against alloantigen, tepezalin at various concentrations was tested in MLR proliferation assays. The assay was set up by stimulating

C57BL/6J (H-2^b) mouse spleen cells with irradiated B6D2F₁/J (H-2^{ab}) mouse spleen cells. As shown in Figure 1, tepezalin inhibited cell proliferation in a dose-dependent fashion with an IC₅₀ of 1.3 μ M. The inhibitory effect was not related to cell toxicity. Tepezalin at concentrations of 25 μ M or less did not affect the viability of anti-CD3 stimulated mouse spleen cells after 24 hr of treatment (Table 1). Since tepezalin is a dual CO/LO inhibitor (10), the possible link of its suppression of MLR proliferation to its inhibition of CO and/or LO was studied. To address this question, the well-known CO inhibitor naproxen and the LO inhibitor zileuton were tested in parallel at doses 10-fold higher than their IC₅₀ for suppression of CO or LO in mice, respectively. Neither of these compounds, nor the combination of both of them, had an inhibitory effect on MLR proliferation (Fig. 1).

To further understand the mechanism of action of tepezalin, the kinetics of tepezalin in inhibiting MLR proliferation was compared to that of the known immunosuppressant, CaA. As shown in Table 2, the inhibitory effect was not diminished when tepezalin was added 24–72 hr after the initiation of MLR. In contrast, CaA was effective only if it was added at the beginning of the cocultures. To determine whether tepezalin and CaA were synergistic in inhibiting MLR proliferation, the two agents were tested in combination. Tepezalin at 0.5 μ M, 1 μ M, or 2 μ M was tested in combination with varying concentrations of CaA (Fig. 2). CaA alone inhibited the response in a dose-related manner with an IC₅₀ of 22 nM. Tepezalin alone inhibited proliferation by 26% at 0.5 μ M, by 55% at 1 μ M, and by 87% at 2 μ M. When tepezalin and CaA were present at suboptimal concentrations, the inhibition was clearly additive. This additive effect was less significant at concentrations of the two drugs that were strongly inhibitory on their own.

Suppression of GVH responses by tepezalin. The immunosuppressive effect of tepezalin as demonstrated in MLR as-

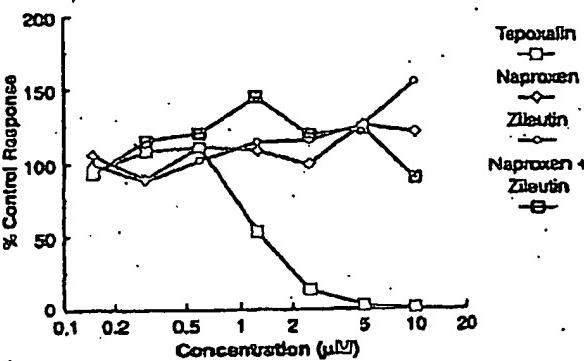


FIGURE 1. Inhibition of MLR proliferations by tepezalin. Spleen cells from C57BL/6J mice were cocultured in triplicate wells with irradiated spleen cells from B6D2F₁/J mice as described in Materials and Methods. Varying concentrations of tepezalin, naproxen, zileuton, or naproxen + zileuton were added to the cultures at the initiation of cultures. ^{3}H -thymidine uptake was measured on day 5. Control cultures contained DMSO diluted in a manner similar to that of the compounds. Uptake of ^{3}H -thymidine in vehicle controls was about 90,000 cpm. Percentages of control responses are calculated as percentages of (cpm of treated cultures/cpm of vehicle controls).

TABLE 1. The effective doses of tepezalin in immunosuppression is not toxic to cells^a

Tepezalin (μM)	Cell viability ^b
100	52.0%
50	76.4%
25	96.5%
12.5	103.0%
6.25	115.5%
3.12	118.4%
1.56	138.5%

^a Viability of anti-CD3 stimulated C57BL/6J spleen cells treated with tepezalin for 24 hr was tested in the MTT viability assay.

^b Cell viability is presented as the percentage of viable cells in tepezalin treated sample compared with that treated with an equivalent amount of the vehicle, DMSO.

TABLE 2. Inhibitory effect of tepezalin and CaA on MLR proliferations (% control response)^c

Concentration (μM)	Time of Treatment				
	0 hr	24 hr	48 hr	72 hr	
Tepezalin	1.25	64.0	52.0	67.0	30.8
	2.5	17.2	12.7	19.2	15.1
	5.0	4.7	5.2	7.8	8.0
Cyclosporine	0.021	40.6	65.0	97.1	133.6
	0.042	18.6	99.5	93.2	148.0
	0.084	5.7	64.1	84.3	119.2

^c Different concentrations of compounds added in MLR cultures at different time points were studied. MLR assays were set up as described in Materials and Methods. The MLR proliferations treated with compounds were compared with their vehicle controls. ³H-thymidine uptake by proliferating cells in MLR assays was measured. Percentages of control responses are calculated as percentages of cpm of treated cultures/cpm of vehicle controls.

says suggests its potential use as an immunosuppressant in clinical therapy. This possible application was verified with *in vivo* murine models of transplantation. A local GVH response was performed by injecting spleen cells from the parental C57BL/6J (H-2^b) mice into the footpads of B6D2F1/J (H-2^{b/d}) mice. GVH responses were demonstrated by the enlargement of the draining popliteal lymph nodes in recipient mice. The lymph nodes of recipient mice increased significantly by day 2 and continued to increase in size with time. The degree of the local GVH response was measured by weighing the draining popliteal lymph nodes. The lymph nodes of tepezalin-treated mice did enlarge on day 2 but did not change significantly later on. After 7 days of the local GVH response, lymph nodes from tepezalin-treated mice were slightly hyperplastic, but were significantly less so than that of the untreated controls (Fig. 3A). GVH responses in mice administered tepezalin orally at 12–50 mg/kg/day were reduced by about 40% of that in the positive control group. Consistent with the findings in mice, tepezalin was also effective in rats, with a 30% suppression of this local GVH response at 12 mg/kg/day (data not shown). The immunosuppressive agent CaA administered subcutaneously to mice at 50 and 75 mg/kg/day was shown to suppress GVH response by 42% and 71%, respectively (Fig. 3B). The results suggest that the immunosuppressive effect of tepezalin at 12 mg/kg/day is comparable to that of CaA at 50 mg/kg/day. To assess whether the inhibitory effect of tepezalin on GVH responses

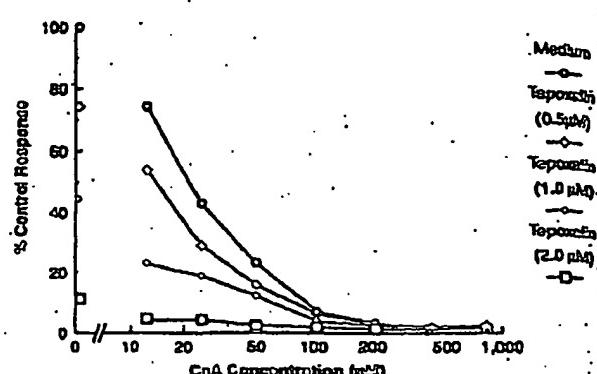


FIGURE 2. Additive inhibitory effects of tepezalin and CaA in MLR proliferations. Proliferation of C57BL/6J mouse spleen cells after 5 days stimulation with irradiated B6D2F1 spleen cells in medium containing tepezalin at 0.5 μM , 1 μM , and 2 μM plus varying concentrations of CaA was assayed as described in Materials and Methods. The proliferative response in cultures containing no drugs was 80,000 cpm.

could be obtained with other CO or LO inhibitors, naproxen and zileuton were again tested in GVH assays. No inhibition was seen with zileuton, naproxen, or a combination of the two compounds (Fig. 3C).

Since tepezalin appears to act late in MLR assays, the effect of tepezalin administered early and late in GVH responses was also studied. Similar to the findings in MLR proliferations, tepezalin given to mice for a minimum of 3 days was sufficient to suppress GVH responses to an extent similar to those treated with tepezalin throughout the 7-day course of the GVH response (Fig. 4). This short treatment with tepezalin could be at the early (day -1 to day 1 or 4) or the late (day 4 to day 6) stage of the GVH response. The inhibitory effect of tepezalin at the late stage of immune responses suggests its mechanism of action to be different from that of CaA. The possible synergism in immunosuppression by tepezalin and CaA was therefore studied in GVH assays. A much stronger suppression of the GVH response was indeed found in mice treated with both tepezalin and CaA rather than those treated with either one of the two drugs (Fig. 5). This synergistic effect was particularly significant when a low dose of tepezalin (6 mg/kg/day) was combined with CaA.

Prolongation of skin allograft survival by tepezalin. The time course of skin allograft rejection in mice is affected by the efficiency of the following two mechanisms: (1) the activation of T cells through recognition of specific alloantigen, and (2) the effector mechanisms mediating tissue destruction. To study the effect of tepezalin on skin allograft survival, experimental allograft rejection was performed by grafting allogeneic BALB/cByJ (H-2^d) mouse tail-skin onto C57/Hol (H-2^b) recipient mice. For the first 6 days after transplantation, allografts appeared normal and their gross appearance was not different from that of syngeneic grafts. The rejection process became apparent by day 6, with signs of swelling and erythema, and quickly culminated into complete graft necrosis. Different doses of tepezalin were tested in skin graft rejection assays. As shown in Figure 6, rejection of allografts

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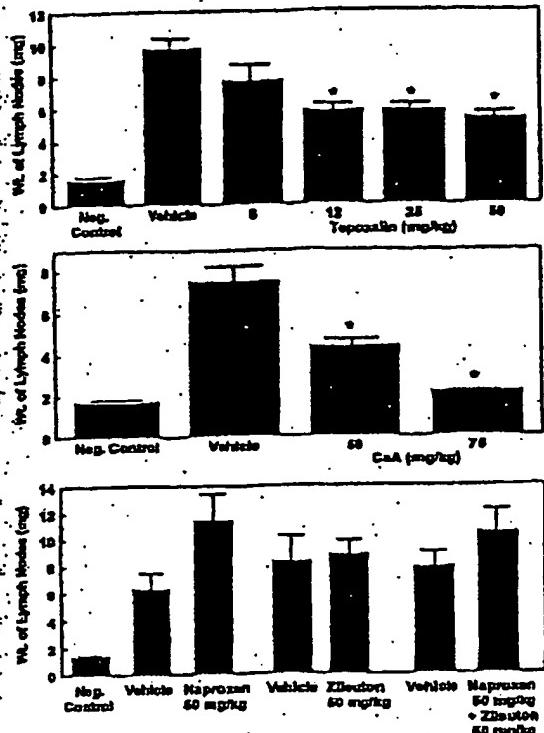


FIGURE 3. Suppression of GVH responses by tepezolin. A local GVH response was triggered by subcutaneous injection of parental C57BL/6J spleen cells into footpads of B6D2F₁ mice, and was measured by weighing the draining lymph nodes as described in Materials and Methods. Mice injected with saline were used as negative controls. Drugs were given to mice from day -1 to day 6 of the GVH response. (A) GVH responses in mice administered different doses of tepezolin or vehicle control (0.5% methylcellulose) orally. Ten mice were used per group. The values from mice treated with tepezolin at 12, 25, and 50 mg/kg/day are significantly different from the vehicle control group (Dunnett's test). Similar group results were obtained from more than three repeated experiments. (B) GVH responses in mice given CaA (50 and 75 mg/kg) or vehicle control (saline) subcutaneously. Five mice were used per group. (C) GVH responses in mice given naproxen, sileuton, or the combination of the two drugs at 50 mg/kg/day orally. Mice as vehicle controls for naproxen were treated with equivalent volumes of 0.5% methylcellulose; for sileuton, they were treated with 50% polyethylene glycol 200; and for the combination of drugs, they were treated with both 0.5% methylcellulose and 50% polyethylene glycol 200. Five mice were used per group. The column bars represent the standard errors. Asterisks indicate a *P* value of <0.05.

in the placebo-treated group started on day 7. About 50% of the allografts in the placebo group were rejected on day 10. Tepezolin at doses of 12.5 and 25 mg/kg/day did not have a significant effect in prolonging graft rejection. When tepezolin at 50 mg/kg/day was administered to mice, a significant prolongation of skin graft rejection was observed. The median survival time of skin grafts, defined as the time point at which 50% of the grafts are rejected, was 10.5 days in the

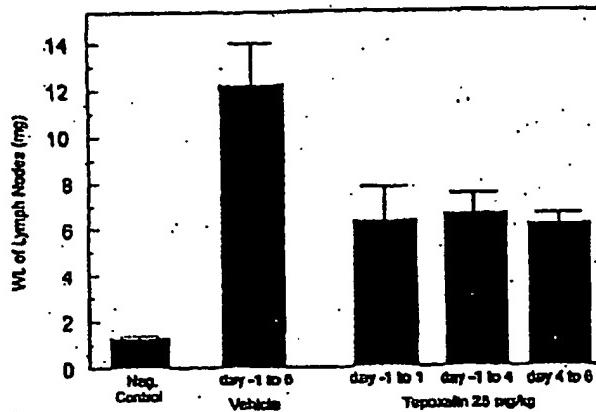


FIGURE 4. Effective suppression of mouse GVH response by short treatments with tepezolin. The GVH response was induced by injection of C57BL/6J spleen cells into the footpads of B6D2F₁ mice and was measured by weighing the draining lymph nodes as described in Materials and Methods. Mice injected with saline instead of spleen cells were used as negative controls. Tepezolin (25 mg/kg) was administered orally to mice at different time schedules as shown. GVH responses in mice treated with vehicle (0.5% methylcellulose) were used as positive controls. Five mice were used per group. The column bars represent the standard errors.

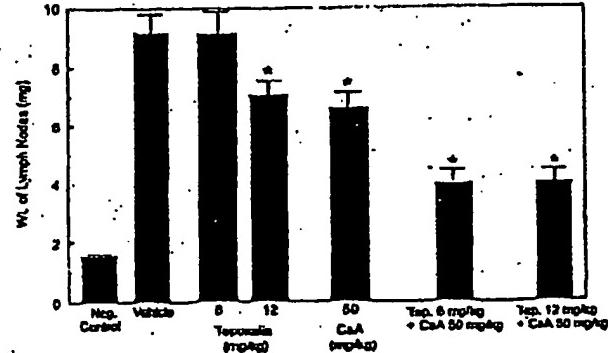


FIGURE 5. Synergistic suppression of mouse GVH responses by tepezolin and CaA. The GVH response was induced by injection of C57BL/6J spleen cells into the footpads of B6D2F₁ mice and was measured by weighing the draining lymph nodes as described in Materials and Methods. Mice were treated with CaA (50 mg/kg) or tepezolin (6 or 12 mg/kg) alone, or the combination of tepezolin (6 or 12 mg/kg) and CaA (50 mg/kg). Mice injected with spleen cells and treated with vehicles were used as positive controls. Mice injected with saline instead of spleen cells were used as negative controls. Twenty mice were used per group. The column bars represent the standard errors. Asterisks indicate a *P* value of <0.05. Similar results were obtained from repeated experiments.

placebo-treated group and was 15.0 days for the group of mice treated with tepezolin at 50 mg/kg/day (*P*<0.05). Furthermore, a combination of tepezolin and CaA at low doses showed a dramatic prolongation of allogeneic skin graft rejection (Fig. 7). About 52% of the mice treated daily with

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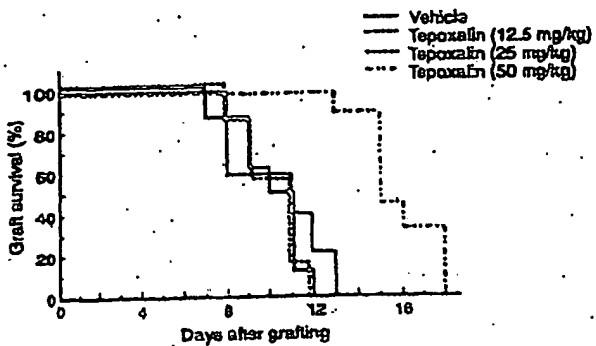


FIGURE 6. Prolongation of skin graft rejections by tepoxalin. BALB/cByJ mouse tail skin was grafted onto the tail of C3H/HeJ mice and rejection of the grafted skin was scored as described in Materials and Methods. Different doses of tepoxalin were administered orally to C3H/HeJ recipient mice the day before and after skin transplantation, and then daily until skin grafts were rejected. Mice given the vehicle (0.5% methylcellulose) orally were used as controls. About ten mice were used per group. Data presented were taken from one of the three repeated experiments. Results obtained from all three experiments were similar. Prolongation of skin rejection in mice treated with tepoxalin 50 mg/kg was significant ($P < 0.05$, Dunnett's test).

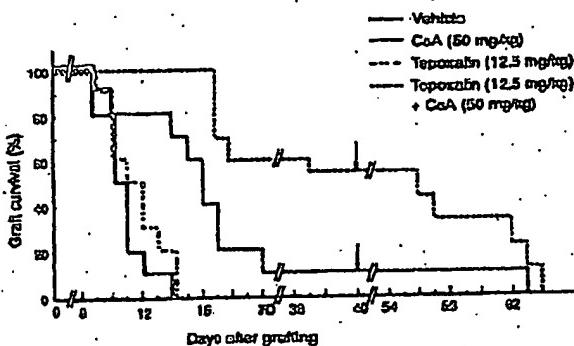


FIGURE 7. Enhanced prolongation of allogeneic skin graft rejection by tepoxalin and CaA. BALB/cByJ mouse tail skin was grafted onto the tail of C3H/HeJ mice as described in Materials and Methods. Tepoxalin (12.5 mg/kg) alone, CaA (50 mg/kg) alone, or tepoxalin (12.5 mg/kg) plus CaA (50 mg/kg) were administered to C3H/HeJ mice the day before and after skin transplantation, and then daily until skin grafts were rejected. Tepoxalin was given orally and CaA was given subcutaneously. For recipient mice with skin grafts surviving for more than 40 days, drug administration was discontinued from day 40, as shown by arrows. About ten mice were used per group. Enhanced prolongation of skin rejection was also observed for the combination of tepoxalin (25 mg/kg) and CaA (50 mg/kg) (data not shown).

tepoxalin (12.5 mg/kg/day) and CaA (50 mg/kg/day) retained the allogeneic skin grafts on day 40 after transplantation. To determine whether immunotolerance to skin grafts is generated by the combined drug treatment, drug dosing was discontinued after day 40 of transplantation. Skin graft rejection was noticeable on day 40 and all the grafts were rejected

on day 24 after drug cessation (Fig. 7). The results suggest that the combination of tepoxalin and CaA potentiates the immunosuppressive effect, but does not induce immunotolerance to the grafts.

DISCUSSION

In this report, we demonstrate that tepoxalin is effective in suppressing the immune responses in murine models of GVH reaction and allogeneic skin graft rejection. This immunosuppressive activity is not seen with other inhibitors of CO or LO.

To study the mechanism of immunosuppression by tepoxalin, we used the *in vitro* mixed lymphocyte reaction, which measures the proliferative response of parental strain C57BL/6J spleen cells when stimulated by B6D2F₁/J spleen cells. Tepoxalin inhibited the alloantigen-driven proliferative response in a dose-related manner with an IC₅₀ of 1.8 μ M and a complete inhibition at 5 μ M. A similar inhibition was seen with CaA, which had an IC₅₀ of approximately 22 nM and a complete inhibition at about 200 nM. However, there were differences in the kinetics of the inhibitions seen with the two compounds. Tepoxalin exerted the same degree of inhibition if added any time up to 72 hr after the set-up of MLR cultures. IL-2 production by T cells occurs early following activation, reaching peak levels by 24 hr of culture (18, 19). CaA has been known for its inhibitory effect on IL-2 production (7, 20, 21) and is therefore expected to affect T cells during the first 24 hr of activation. The fact that tepoxalin inhibits proliferation late in MLR assays suggests its inhibition of later events in T cell activation. One possibility is that the IL-2-mediated signal transduction pathway is affected by tepoxalin, which has been shown on human lymphocytes in our previous report (13).

GVH disease is a common problem in bone marrow transplantation that leads to frequent morbidity and mortality (22). Skin grafts trigger strong immune responses and have been one of the most difficult grafts in transplantation (3). The immunosuppressive activity of tepoxalin was demonstrated in murine models of GVH responses and allogeneic skin graft rejections. Tepoxalin was found to inhibit GVH responses at 12 mg/kg/day and to prolong skin graft rejections at 50 mg/kg/day. The possibility that tepoxalin blocks a later event in immune response is again implicated by its suppression of GVH reaction even when it was administered to mice 4 days after the initiation of the response.

Tepoxalin is known to be a dual CO and LO inhibitor with potent antiinflammatory effects (10). One of the obvious questions to ask is whether its immunosuppression is due to the inhibition of the CO or LO enzymes. The involvement of CO and LO in the modulation of immune responses remains controversial. Arachidonic acid metabolites produced by these enzymes, such as prostaglandins and leukotrienes, have many biological activities, including the modulation of inflammation and immune response (23-29). Indeed several inhibitors of LO have been shown to prolong graft rejection in transplantation (30-33). However, it was noticed that those LO inhibitors with immunosuppression activity are also potent antioxidants with inhibitory effects on NF- κ B activity (34, 35). Therefore the immunoregulatory effects of these

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suggested that compounds may not be directly related to inhibition of LO. We compared the effect of tepezalin with other known LO/CO inhibitors or cyclooxygenase inhibitors in our studies. Naproxen (CO inhibitor) or zileuton (5-lipoxygenase inhibitor), or their combination, did not have any effect on MLR proliferations or GVH responses. We have reported recently that tepezalin is distinct from other CO and LO inhibitors in its inhibition of NF- κ B activities (14). NF- κ B is a constitutive heterodimeric transcription factor that is a potent mitotropic transactivator of many target genes involved in immune or inflammatory responses (16, 17). The immunosuppressive effect of tepezalin may be attributed to its inhibition of NF- κ B and not related to the general inhibition of CO or 5-lipoxygenase activity.

Taken together, these data show that tepezalin is an effective immunosuppressive agent. Since the mechanism of tepezalin appears to be different from CsA in immunosuppression, it suggests a possible combinational use of the two compounds in immunosuppressive therapy. Moreover, tepezalin is devoid of ulcerogenic actions in gastrointestinal systems that are the common side effects of other NSAID on way (11, 12). The LD₅₀ of tepezalin in mice and rats was more than 400 mg/kg, which is over 10-fold higher than the effective doses used in *in vivo* immunosuppression. Tepezalin could therefore be an important addition to the existing immunosuppressive therapeutic drugs to enhance the efficacy of treatment and to reduce drug toxicity in transplantation and autoimmunity.

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COMBINED THERAPY WITH INTERLEUKIN-4 AND INTERLEUKIN-10 INHIBITS AUTOIMMUNE DIABETES RECURRENCE IN SYNGENEIC ISLET-TRANSPLANTED NONOBES DIABETIC MICE

ANALYSIS OF CYTOKINE mRNA EXPRESSION IN THE GRAFT¹

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Syngeneic pancreatic islet grafts in nonobese diabetic (NOD) mice elicit a cell-mediated autoimmune response that destroys the insulin-producing β cells in the islet graft. IL-4 and IL-10 are cytokines that inhibit cell-mediated immunity. In this study, we evaluated the effects of IL-4 and IL-10 on the survival of syngeneic pancreatic islets transplanted into diabetic NOD mice. Islet grafts survived beyond 18 days and normoglycemia was maintained in 87% (10 of 15) of mice treated with IL-4 plus IL-10, but in none (0 of 20) of vehicle-injected (control) mice. Also, 40% (6 of 15) of the mice treated with IL-4 plus IL-10 were normoglycemic at 30 days after transplantation, compared with 14% (1 of 7) of the mice treated with IL-4 alone, 8% (1 of 13) of the mice treated with IL-10 alone, and none (0 of 20) of the control mice. Histological examination of grafts at 10 days after transplantation revealed perisit accumulations of mononuclear leukocytes and intact islet β cells in grafts from IL-4 plus IL-10-

treated mice, whereas islets were infiltrated by leukocytes and the β cell mass was greatly reduced in grafts from control mice. Polymerase chain reaction (PCR) analysis of cytokine mRNA expression in the grafts revealed higher levels of IL-2, IFN γ , and IL-10 mRNA in grafts of diabetic compared with normoglycemic control mice, whereas IFN γ and TNF α mRNA levels were significantly decreased in grafts of IL-4 plus IL-10-treated mice compared with either normoglycemic or diabetic control mice. These results suggest that T helper (Th)1 cells and their cytokine products (IL-2, IFN γ , and TNF α) may promote islet β cell destructive insulitis and autoimmune diabetes recurrence in syngeneic islet-transplanted NOD mice, and that administration of IL-4 plus IL-10 may inhibit diabetes recurrence by suppressing Th1 cytokine production in the islet grafts.

Insulin-dependent diabetes mellitus (IDDM)² results from destruction of the insulin-producing pancreatic islet β cells by the host's own immune system. Whereas it is not known what may initiate this autoimmune response against islet β cells, there is abundant evidence that IDDM is T cell-dependent (1, 2). However, it is unclear which T cells are involved and how they may lead to islet β cell destruction. A variety of immune/inflammatory cells infiltrate the pancreatic islets and constitute the insulitis lesion (3, 4). There is evidence in human patients with IDDM (5-8) and in animals with spontaneous IDDM resembling the human disease—the nonobese diabetic (NOD) mouse and the biobreeding (BB) rat (9-22)—that islet β cell destruction may involve heterogeneous effec-

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⁹ Abbreviations: BB, Biobreeding; CPA, complete Freund's adjuvant; IDDM, insulin-dependent diabetes mellitus; NOD, nonobese diabetic; PCR, polymerase chain reaction; Th, T helper.

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Granulocyte Macrophage Colony-Stimulating Factor Improves Survival in Two Models of Gut-Derived Sepsis by Improving Gut Barrier Function and Modulating Bacterial Clearance

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Objective

The effect of recombinant murine granulocyte macrophage colony-stimulating factor (rmGM-CSF) on survival and host defense was studied using two clinically relevant models of infection that included transfusion-induced immunosuppression.

Summary Background Data

Granulocyte macrophage colony-stimulating factor improves resistance in several models of infection, but its role in transfusion-induced immunosuppression and bacterial translocation (gut-derived sepsis) has not been defined.

Methods

Balb/c mice were treated with 100 ng of rmGM-CSF or placebo for 6 days in a model of transfusion, burn, and gavage, or cecal ligation and puncture (CLP). Translocation was studied in the first model.

Results

Survival after transfusion, burn, and gavage was 90% in rmGM-CSF-treated animals versus 35% in the control group ($p < 0.001$). After CLP, survival was 75% in the rmGM-CSF group versus 30% in the control group ($p = 0.01$). Less translocation and better killing of bacteria was observed in the tissues in animals treated with rmGM-CSF.

Conclusion

The ability of rmGM-CSF to improve gut barrier function and enhance killing of translocated organisms after burn injury-induced gut origin sepsis was associated with improved outcome. Granulocyte macrophage colony-stimulating factor also improved survival after CLP.

Infectious complications continue to be a major cause of morbidity and mortality in surgical patients despite improved intensive care techniques, multimodality therapy, and preoperative support.¹ Fatal outcome from sepsis usually results from progressive multiple organ failure or from septic shock.^{1,2}

Critically ill surgical and polytraumatized patients have numerous disturbances of cellular and humoral immunity, leading to a depression of defense against infections.^{3,4} Impairment of the ability of polymorphonuclear leukocytes (PMN) to phagocytize and kill bacteria are the most important alterations found. In burn injury, the development of acquired defects of host defense are well documented, particularly neutrophil function and its relationship to sepsis.⁵ In patients with large burns, there commonly is a severe depression of functional humoral and myeloid components of host defense.^{5,6} Moreover, it is suspected that intestinal flora can translocate from the intestinal tract and cause complicating systemic infections in critically ill and injured patients.

The barrier function of the epithelial layer of the mucosa and the killing capacity of the opsonophagocytic system influence the numbers of viable bacteria in the tissues associated with the translocation process. A deficiency of either one of these after trauma could predispose to the biological consequence of translocation of microbes or their products, e.g., stimulation of the cytokine response. Berg et al.⁷ and Deitch et al.⁸ popularized the concept of translocation in the 1980s. These investigators and others have demonstrated the appearance of enteric bacteria in the mesenteric lymph nodes (MLN), liver, and spleen after 1 to several days in a number of conditions, including bacterial overgrowth,⁹ hemorrhagic shock,¹⁰ thermal injury,¹¹ intestinal obstruction,¹² endotoxemia,¹³ intravenous hyperalimentation,¹⁴ and antibiotic therapy.¹⁵ Microbial translocation subsequently was shown to occur directly through intact enterocytes,¹⁶ although bacteria may invade the gut directly through ulcerations.¹³ Because bacterial translocation contributes to adverse outcomes rather than being a passive associated event,¹⁷ prevention of this process could become an important therapeutic tool for the management of critically ill patients.

This study assessed the potential effects of rmGM-CSF on prevention of bacterial translocation and outcome (survival) after injury in two different types of experimental sepsis. Granulocyte macrophage colony-stimu-

lating factor was used because it has been reported to enhance numerous functional activities associated with host defense.¹⁸⁻²⁴

MATERIALS AND METHODS

Animals and Animal Care

Adult female Balb/c mice (H-2^d) (Charles River Laboratories, Wilmington, MA), weighing 18 to 22 g, and adult female C3H/HeJ mice (H-2^k) (Jackson Laboratory, Bar Harbor, ME) were used in these experiments. The mice were quarantined for 1 week to allow adaptation to the environment and to exclude any animals with pre-existing diseases. During this period, and throughout these experiments, the mice were allowed free access to water and food (Rodent Laboratory Chow 5001, Purina Mills, Inc., St. Louis, MO). The protocol was approved by the University of Cincinnati Medical Center's Institutional Animal Care Use Committee, and the animals were housed in an American Association for the Accreditation of Laboratory Animal Care (AAALAC) approved facility. All investigations adhered to the guide for the Care and Use of Laboratory Animals as set forth by the Committee on the Care and Use of Laboratory Animals, the National Research Council, the United States Department of Health and Human Services, and the National Institutes of Health.

Blood Harvesting and Transfusion Procedures

Under methoxyflurane anesthesia, C3H/HeJ mice were bled by cardiac puncture. The harvested blood was mixed at a 7:1 volume ratio with anticoagulant citrate phosphate dextrose adenine solution (Fenwal Laboratories, Deerfield, IL) and stored at 4°C overnight. Five days before burn injury, Balb/c mice were transfused through a tail vein with 0.2 mL C3H/HeJ blood to induce a mild but well-defined immunosuppression.²⁵

Preparation of rmGM-CSF and Animal Treatment

Recombinant murine granulocyte macrophage colony-stimulating factor (R&D Inc. Systems, Minneapolis, MN, lot No. BJ091.12, 25 µg) was supplied in powder form. It was reconstituted from lyophilized material and stored at -70°C at a concentration of 2 µg/mL in phosphate buffered saline (PBS) with 0.1% bovine serum albumin. Immediately before use, rmGM-CSF was diluted to a concentration of 500 ng/mL in sterile PBS (pH 7.4).

At the time of treatment, animals received 100 ng of rmGM-CSF in 0.2 mL of PBS, injected subcutaneously

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in the nape of the neck or an equal volume of sterile PBS for the control group. Mice were injected at 8-hour intervals for 6 days preburn or pre-CLP.

White Blood Cell Counts

Balb/c mice were anesthetized with methoxyflurane and blood was collected by cardiac puncture and placed into micro test tubes with ethylenediaminetetraacetic acid (Becton Dickinson and Co., Cockeysville, MD). White cell counts were performed using a hemocytometer (Hausser Scientific, Horsham, PA) and differential cell counts were performed using a blood film smear stained with Diff-Quick (Baxter, McGaw Park, IL) and examined under a light microscope. A total of 100 cells were counted and classified according to morphology into neutrophils (segmented and unsegmented), lymphocytes, monocytes, eosinophils, and basophils.

Isolation of Splenic Macrophages

Mice were killed and spleens were removed aseptically and placed in Hanks' balanced salt solution (HBSS) (Gibco, Grand Island, NY) containing 1% penicillin/streptomycin solution (Sigma Chemical Co., St. Louis, MO) but without calcium and magnesium. The spleens were minced using disposable sterile scalpels and filtered through nylon glass wool to remove debris. The cells were washed with HBSS and centrifuged at 1500 rpm for 10 minutes at 4°C. Red blood cells were then lysed using 10 mL of sterile water and 10 mL of 2X (concentrated) HBSS without calcium; magnesium was added after 10 seconds. The cells were washed with HBSS and centrifuged two more times and then counted. The numbers of total white cells and macrophages were determined using a hemocytometer with a gentian violet stain.

CLP Procedure

Using aseptic techniques, Balb/c mice were anesthetized with methoxyflurane and given a minimal midline laparotomy to expose the cecum. The cecum was then ligated with a silk suture and punctured once with a 25-gauge needle according to a technique described previously.²⁶

Preparation of ^{111}In Oxine-Labeled *Escherichia coli*

Escherichia coli (Stock #53104, University of Minnesota, Minneapolis, MN) was inoculated into 25 mL brain heart infusion broth (Baltimore Biological Laboratories, Baltimore, MD) and incubated overnight at 37°C in a shaking water bath. This culture was centrifuged at

4000 rpm for 10 minutes and washed twice in sterile saline. The pellet was resuspended in sterile saline, 1.0 mCi of ^{111}In oxine (Syncor, Cincinnati, OH) was added, and the suspension then was incubated for 40 minutes at 37°C. The isotope-labeled bacteria were washed, resuspended in sterile saline, and adjusted to a final concentration of 1×10^{10} microorganisms/0.2 mL using a Klett densitometer (Klett Manufacturing Co., Long Island, NY).

Gavage and Burn Procedures

One day before burn, the hair of animal torsos was removed by clipping. Food was withheld for 18 hours, but water was provided ad libitum before gavage with 0.2 mL of ^{111}In *E. coli* while the animals were awake. After gavage, they were anesthetized with methoxyflurane inhalation, and a 20% full-thickness flame burn was inflicted using the technique of Stieritz and Holder.²⁷ Saline (0.2 mL) was given intraperitoneally immediately after burn injury for fluid resuscitation, and the animals were allowed to recover from anesthesia with free access to food and water.

Quantitation of Bacterial and Radionuclide Counts

The solid tissues were weighed individually and homogenized in 1.0 mL of sterile normal saline. One hundred microliters of the homogenate were removed and plated on eosin methylene blue (EMB) plates (Becton Dickinson and Co., Cockeysville, MD) for quantitative colony counts after 24 hours of aerobic incubation at 37°C. The number of colony forming units (CFUs) were counted and expressed as CFU per gram of tissue by multiplying the actual counts by:

$$\frac{(1 + \text{weight}) \times 10}{\text{weight}}$$

To ensure that the *E. coli* grown from the tissues was the same species as the gavaged *E. coli*, the Sceptor System Test #80401 (Becton Dickinson, Towson, MD) was used. This system employs a combination of antimicrobial susceptibility tests, biochemical identification, and a β -lactamase test to identify specific species of enteric bacteria.

The remaining portions of homogenates were used to determine radionuclide counts (as measured by degradation per minute (dpm)). The counts were determined using a gamma counting system (Beckman Model Gamma 5500, Beckman Instruments Inc., Fullerton, CA). The radioactivity in tissues was expressed as dpm/g of tissue. We determined that 1 dpm count corresponded to ap-

proximately 11 viable bacteria (this value was obtained by counting the radionuclide and bacterial counts in serial dilutions of the gavaged suspension) as measured by a Klett densitometer. The percentage of surviving bacteria in tissues was calculated by the following formula:

$$\frac{\text{CFU/g}}{\text{dpm/g} \times 11} \times 100$$

STATISTICAL ANALYSIS

To achieve a normal distribution of the data, the dpm values were converted to a logarithmic scale (10^{10}). Statistical analysis for radionuclide counts, the number of CFUs, and the percentage of viable bacteria was performed with the Wilcoxon rank sum test. Survival rates were analyzed by chi square test. White cell counts were analyzed by Student's t test. The difference among the means was considered significant if the p value was <0.05 .

Experimental Design

All of the following experiments were repeated at least once to demonstrate consistency, and the results present data pooled from replicate studies.

Experiment I

To test the *in vivo* activity of rmGM-CSF, two initial groups of Balb/c mice were randomized to receive 100 ng of rmGM-CSF (n = 7) or sterile PBS (n = 7) three times daily for 6 days. The animals then were transfused with allogeneic blood (from C3H/HeJ mice). Five days after transfusion, under methoxyflurane inhalation, blood was collected by cardiac puncture, and white cell counts were performed. The animals were killed, and the spleen was removed aseptically to determine the number of total white cells and macrophages.

Experiment II

Two groups of Balb/c mice were randomized to receive 100 ng of rmGM-CSF (n = 20) or an equal volume of sterile PBS (n = 20) every 8 hours for 6 days. Then animals were transfused with allogenic blood (from C3H/HeJ mice). Five days after transfusion, the mice were gavaged with 10^{10} *E. coli* followed immediately by a 20% total body surface area thermal injury. Survival was observed for 10 days after burn.

Experiment III

Two groups of Balb/c mice were treated with 100 ng of rmGM-CSF (n = 20) or an equal volume of sterile PBS (n = 20) every 8 hours for 6 days. Then the animals were transfused with allogenic blood (from C3H/HeJ mice). Cecal ligation and puncture were performed 5 days after transfusion, and the animals were observed 10 days for survival.

Experiment IV

An additional 30 animals underwent the same procedure as described in Experiment II, except they were gavaged with 10^{10} ^{113}In oxine-radiolabeled *E. coli* in 0.2 mL and killed 4 hours after burn. The animals' abdomens were prepped with 70% alcohol, and by a midline laparotomy, MLN, spleen, and liver were harvested using aseptic techniques.

RESULTS

Experiment I

Peripheral Blood Changes

White cell counts were performed on mice after 6 days of treatment with rmGM-CSF or placebo. As shown in Table 1, there were significant increases in the number of leukocytes ($p < 0.05$) and neutrophils ($p < 0.05$) versus control. Monocytes increased fourfold in treated animals

Table 1. PERIPHERAL BLOOD WHITE CELL CONCENTRATIONS IN MICE INJECTED WITH rmGM-CSF OR STERILE PBS

Group	Total Cells	Differential Cell Counts (Cells/ μl)					
		Neutrophils	Bands	Lymphocytes	Monocytes	Eosinophils	Basophils
rmGM-CSF	5966 \pm 1100*	1705 \pm 265*	42 \pm 22	4031 \pm 1152	169 \pm 152*	11 \pm 10	8 \pm 7
Control	3025 \pm 363	911 \pm 127	11 \pm 11	2047 \pm 257	42 \pm 26	8 \pm 7	6 \pm 5

* $p < 0.05$ (Student's t test).

Values are expressed as mean \pm SEM.

Table 2. EFFECT OF rmGM-CSF ON CELLS IN THE SPLEEN

Group	Total White Cells/g Spleen	Macrophages/g Spleen
rmGM-CSF	20000 ± 2757*	1483 ± 327*
Control	10250 ± 1030	818 ± 100

* p < 0.05 (Student's t-test). Values are expressed as mean ± SEM.

compared with nontreated animals ($p < 0.05$). Although the rmGM-CSF group showed a greater number of lymphocytes than the control group, this difference was not significant.

Splenic Changes

Total white cell counts in mice injected with rmGM-CSF were significantly higher than in PBS-injected mice ($p < 0.05$; Table 2). Differential cell counts in rmGM-CSF treated animals revealed a consistent rise in the percentage of macrophages compared with nontreated animals ($p < 0.05$).

Experiment II

Survival After Transfusion, Bacterial Gavage, and Burn Injury

Mice treated with rmGM-CSF had a 90% survival (18/20); however, in nontreated animals, survival was 35% (7/20) ($\chi^2 = 10.667$, $p < 0.001$). Death occurred within the first 48 hours (Fig. 1).

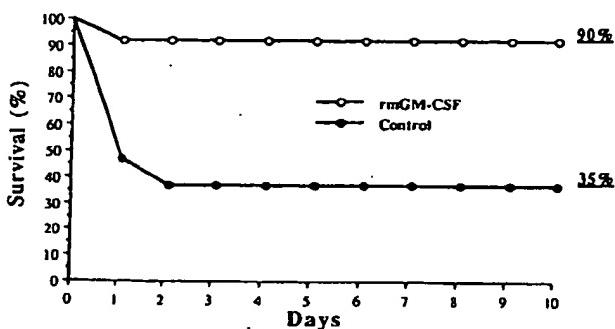


Figure 1. Survival rate of transfused, gavaged, and burned mice treated with rmGM-CSF 100 ng X 3/day for 6 days and nontreated animals. (rmGM-CSF vs. control; $\chi^2 = 10.667$, $p < 0.001$ [Chi square test]).

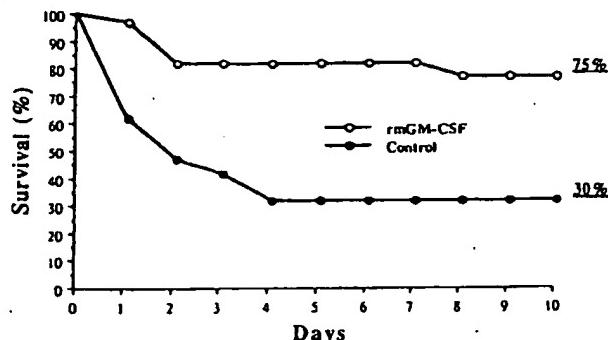


Figure 2. Survival rate after transfusion and cecal ligation and puncture. (rmGM-CSF vs. control; $\chi^2 = 6.416$, $p = 0.01$ [Chi square test]).

Experiment III

Survival After Transfusion and CLP

The survival rate of the animals treated with rmGM-CSF that received allogenic transfusion was 75% (15/20) versus 30% (6/20) in the control group (Fig. 2). ($\chi^2 = 6.416$, $p = 0.01$).

Experiment IV

Translocation and Bacterial Survival

The magnitude of translocation of ^{111}In *E. coli* for the two groups, as measured by dpm/g of tissue, is shown in Figure 3. Nontreated animals had a significantly greater amount of bacteria translocated to the MLN, liver, and

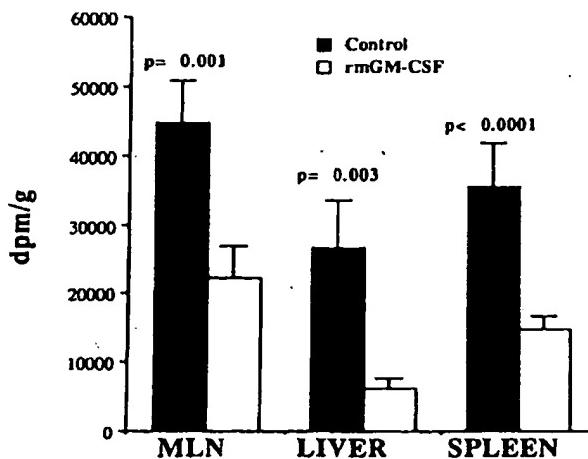


Figure 3. Degree of translocation of ^{111}In -labeled *E. coli* 4 hours after gavage with 10^{10} *E. coli* and burn injury as measured by dpm/g tissues. Data are expressed as mean ± SEM.

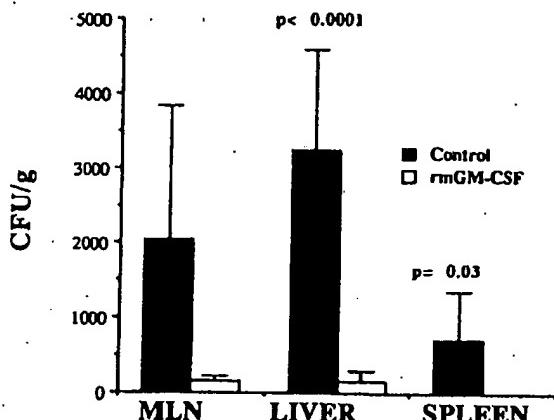


Figure 4. Number of viable *E. coli* as measured by CFUs/g of tissue. Values are expressed as mean \pm SEM.

spleen, compared with rmGM-CSF animals ($p = 0.001$, $p = 0.003$, and $p < 0.0001$, respectively).

The number of viable *E. coli* recovered from the tissues, as measured by CFUs/g, showed that the mice treated with rmGM-CSF had fewer bacteria in MLN, liver, and spleen, compared with control animals (Fig. 4). The rmGM-CSF group showed fewer numbers of viable *E. coli* compared with nontreated mice in all tissues studied, and this difference was significant in the liver and spleen ($p < 0.0001$, $p = 0.03$, respectively, Fig. 4). Moreover, the incidence of viable bacteria in the liver was significantly higher in control animals compared with mice that received rmGM-CSF. Control mice had positive cultures in 93% (14/15) versus only 13% (2/15) in the treated group ($\chi^2 = 16.205$, $p < 0.0001$; Fig. 5)

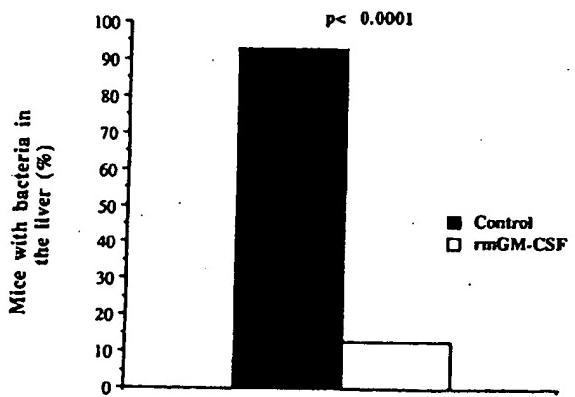


Figure 5. Percentage of mice with bacteria isolated from the liver 4 hours postburn. There was a significant increase in the incidence of bacterial translocation in nontreated mice.

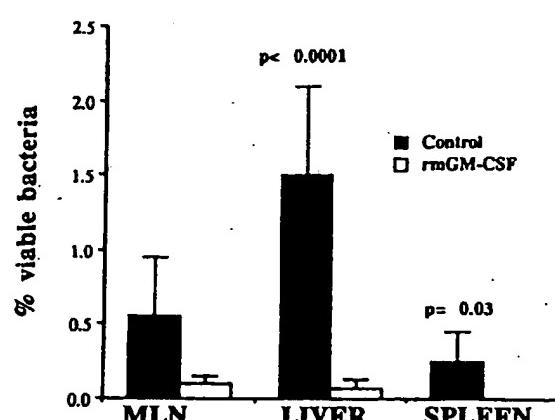


Figure 6. Calculated percentage of translocated bacteria that survived in the tissues. Values are expressed as mean \pm SEM.

The calculated percentage of translocated bacteria that survived showed that the treatment with rmGM-CSF improved the ability to kill bacteria that translocated in the MLN, liver, and spleen, compared with controls (Fig. 6), but this was significant only for the liver and spleen ($p < 0.0001$, $p = 0.03$).

DISCUSSION

Despite advances in anti-infective therapy, sepsis remains a major cause of mortality in surgical and injured patients. A variety of alterations in the myelopoietic cell system after injury have been demonstrated, including decreased neutrophil chemotaxis and bactericidal activity,²⁸ decreased serum colony-stimulating activity, and a decrease in monocyte release of colony-stimulating factors.²⁹ The occurrence of these multiple defects has been shown to precede the onset of clinical septic events in patients by several days.²⁹

In the last decade, several studies have recognized bacterial translocation as a potential cause of systemic infections,¹⁰ clinical sepsis with undefined focus of infection,³⁰ multiple organ failure,³¹ endotoxemia,¹³ septic syndrome,³¹ and hypermetabolic responses,³² conditions that often complicate severe illnesses and trauma. The severity of infections associated with bacterial translocation is determined by the burden of bacteria and their products crossing the mucosal barrier, and the ability of host defenses to kill and clear microbes and their products, such as endotoxin.

Granulocyte macrophage colony-stimulating factor is a well-characterized specific glycoprotein, purified in the mid 1980s, and subsequently cloned, that participates in the regulation of production, differentiation, and function of two related white cell populations of the blood,

the granulocytes and monocyte-macrophages. Granulocyte macrophage colony-stimulating factor does not stimulate proliferation of mature cells. However, it prolongs the life span of these cells by suppressing apoptosis, which is the mechanism of active self-destruction characterized by specific DNA degradation and morphological changes in cells.³³ Granulocyte macrophage colony-stimulating factor has been shown to be directly chemotactic for neutrophils,¹⁸ but high concentrations lead to migration inhibition,²⁰ probably with a down-regulation of the adhesion molecule LAM-1.³⁴ Several studies have recognized that it also can increase the capacity of neutrophils to phagocytose and kill bacteria, yeast, and parasites^{21,22} to participate in antibody-dependent, cell-mediated cytotoxicity²⁴ through binding the Fc portion of both IgG and IgA¹⁹ with an up-regulation of immunoglobulin receptors on the surface of phagocytic cells.³⁵ Granulocyte macrophage colony-stimulating factor also primes neutrophils and macrophages so that they respond more readily to major physiological chemoattractants, including complement-derived C3a, leukotriene B₄, and bacterial products, such as N formyl-methionyl-leucyl-phenylalanine, and releases reactive oxygen metabolites (superoxide) that have bactericidal activity.²³ Therefore, this factor could directly improve resistance to infections in critically ill patients by reversing defects of neutrophil-macrophage function or improving their production. Other mechanisms to explain the effects of GM-CSF may occur through other immune-potentiating processes. In fact, GM-CSF has been shown to affect the expression of CR1 and CR3 receptors, expression of class II major histocompatibility complex, and interleukin-1 secretion from macrophages and cloned T-lymphocytes.^{36,37} Moreover, expression of the adhesion molecule CD11b is rapidly up-regulated by exposure of human neutrophils to GM-CSF.³⁸

This study tested the effects of rmGM-CSF on the susceptibility of transfused and burned mice to gut origin sepsis and on the resistance of animals to bacterial peritonitis after CLP. The relationship between rmGM-CSF on bacterial translocation of enteric bacteria and on the ability of the host defense to kill organisms was also determined. For this latter aim, we used a model for evaluating translocated bacteria using a known quantity of ¹¹¹In oxine-labeled *E. coli* inoculated intragastrically by gavage. This model is especially useful because the number of microorganisms translocating across the mucosal barrier and the killing of translocated microorganisms can be studied simultaneously. Moreover, ¹¹¹In oxine is not redistributed by metabolic processes in the organism or in the phagocytic cells after ingestion. This study used a blood transfusion/burn model with a gavage of 10^{10} *E. coli* that is associated with a mortality rate of approximately 65% to 90% during 10 days of observation,²⁵ and

a model of transfusion/CLP with a mortality range between 70% and 100%.³⁹⁻⁴¹ Blood transfusion leads to immunosuppression and increased susceptibility to infections.^{42,43} Because blood transfusions are used commonly in surgical patients, burn injury, and trauma, these models may accurately reflect the pathophysiologic perturbations of critically ill patients. The CLP model was used because the hemodynamic and metabolic effects in rodents²⁶ seem to have clear parallels to the clinical situation of septic shock in humans, as described by MacLean et al.⁴⁴ The high mortality rates of these models also are ideal for the evaluation of therapeutic interventions. This study suggests that the modulation of host bactericidal mechanisms with rmGM-CSF strongly affects mortality in either model of sepsis. These experiments also show improved killing of translocated bacteria in the burn/transfusion model.

Other reports suggest that the administration of rmGM-CSF in animal models potentiates host defense mechanisms. Recently, Molloy et al. showed that treatment with rmGM-CSF in mice with 30% total body surface area thermal injury and induced sepsis significantly improved survival rate as compared with nontreated animals.³⁹ Tanaka et al. reported that administration of rmGM-CSF to leukopenic mice injected with lethal inocula of *Pseudomonas aeruginosa* improved their survival relative to controls, and the numbers of viable bacteria in the blood and in several tissues were significantly lower in treated versus control animals.⁴⁵ Frenk et al. showed that rmGM-CSF administered to neonatal rats 6 hours before a 90% lethal dose challenge of *Staphylococcus aureus* significantly improved survival, and decreased the number of blood cultures positive for the microorganism.⁴² Moreover, other studies in immunocompromised mice treated with GM-CSF showed that mean survival time and survival rate were increased and bacterial burden was reduced compared with control animals after inoculation with lethal doses of different kinds of microorganisms.⁴⁷⁻⁴⁹

The observed therapeutic effects of rmGM-CSF on resistance of bacterial translocation could be caused mainly by the stimulation of an enhanced rate of phagocytosis, by induction of bactericidal or bacteriostatic mechanisms, or by stimulation of an increased production of neutrophils and macrophages by the bone marrow. Examination of the numbers of peripheral blood white cells and total cell counts and macrophages in the spleen in our study revealed significant changes in the treated group compared with control animals (Tables 1,2). Our observations are in agreement with Metcalf et al., who showed that injection of rmGM-CSF into normal mice three times daily for 6 days resulted in an elevation of blood neutrophil levels and spleen content of macrophages.^{50,51} Recently, Faisal et al. showed, as a late

effect, that treatment with rmGM-CSF induced activation and an increase in lymphoid cell number, especially B cells in patients with refractory aplastic anemia.⁵²

Our data showed an unexpected "cytoprotection" activity of rmGM-CSF on the gastrointestinal mucosa, significantly reducing the amount of bacteria that translocated through the gut (Fig. 3) and improving survival (Fig. 1). The exact mechanism for rmGM-CSF-related cytoprotection activity, however, is not clear, but could be related to the production of prostaglandin E (PGE) mediated by GM-CSF. Kurland et al. demonstrated a close biosynthetic relationship between the production of colony-stimulating factors and PGE by macrophages.⁵³ There are several mechanisms by which colony stimulating factors may promote new PGE production. Colony-stimulating factors may activate phospholipase to liberate arachidonate from membrane-bond phospholipids or activate cyclooxygenase directly to convert arachidonate prostaglandin G2 and H2, bioactive precursors of PGE₂. Recently, our group has shown that PGE analogs significantly reduce bacterial translocation.¹⁷ The potential mechanism of action of PGE on the intestinal mucosa has been attributed to various effects, including increased gastrointestinal blood flow, enhancement of mucus secretion, promotion of bicarbonate secretion, and cyclic adenosine monophosphate production.⁵⁴

Receptors for GM-CSF are present on cells other than those of hematopoietic origin, although their presence has not been studied in the gut epithelium. Specific binding of GM-CSF to a receptor has been observed in non-hematopoietic tissues, such as vascular endothelial cells.⁵⁵ Granulocyte macrophage colony-stimulating factor induces vascular endothelial cells to proliferate and migrate both *in vitro* and *in vivo*,^{55,56} suggesting a possible role for GM-CSF in neovascularization after injury or inflammatory disorders of gastrointestinal mucosa.

Our data suggest that the ability of rmGM-CSF to improve survival in two different types of sepsis is related to augmentation of the production and function of granulocytes and macrophages and to an improvement in gut barrier function.

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- F₁ recipient mice were injected intraperitoneally with the NK1.1 monoclonal antibody PK135 to inhibit H₁ barrier rejection (9). Bone marrow of either female BALB/c or C57BL/6 mice was depleted of T cells with monoclonal antibody (mAb) J11 (anti-Thy-1), mAb C3PO (anti-CD2), mAb RL172 (anti-CD4), and mAb 3.155 (anti-CD8). Approximately 2×10^7 to 4×10^7 bone marrow cells were injected into recipient F₁ mice. Four months after the bone marrow reconstitution, one mouse from each of the two bone marrow constructs was killed. Spleenocytes were stained for MHC class II molecules H²P and I^AP with mAb 14.4.4 [immunoglobulin G2a (IgG2a)] and mAb Y-3P (IgG2a), respectively, to confirm complete reconstitution with donor bone marrow. The CT26NT cell line was produced by infection of CT26 with a Moloney-based defective recombinant retrovirus, provided by E. Gibbons (J. V. Fletcher, N. Roy, E. Gibbons, *J. Immunol.* 147, 2637 (1991)), containing the NP genes from the PR8 influenza strain and a neomycin-resistance gene. Infected cultures were selected in G418 (400 µg/ml). Individual colonies were tested for NP expression by NP mAb staining and lysis by NP + H-2^K-specific CTL lines. A clone positive for both antibody staining and lysis was chosen for further experiments. Cells (10^6 CT26NP + 1×10^6 CT26 wild-type or 1×10^6 CT26NP + 1×10^6 CT26-GM-CSF) were irradiated (50 Gy) and injected subcutaneously in the left flank of the chimeras. Spleens were removed from the mice 2 weeks after the initial immunization, and splenocytes were cultured *in vitro* with either NP(147-155) peptide or NP(368-374) peptide in the presence of interleukin-2 and splenocytes from a (BALB/c × C57BL/6)F₁ mouse. After a 7-day *in vitro* incubation, the splenocytes were harvested and plated in triplicate on a 96-well V-bottom microtiter plate at various effector-to-target ratios. Surrogate target cells, P815(H-2^D) and MC3T3G(H-2^D), were labeled with ⁵¹Cr and added to the effector cells (3000 cells/well) in the presence of synthetic NP(147-155) peptide (500 pg/ml) or NP(368-374) peptide (50 pg/ml). After 4-hour incubation of the cell mixture at 37°C and in 5% CO₂, the media were harvested and counted on a gamma counter.
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Cloning of a T Cell Growth Factor That Interacts with the β Chain of the Interleukin-2 Receptor

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A cytokine was identified that stimulated the proliferation of T lymphocytes, and a complementary DNA clone encoding this new T cell growth factor was isolated. The cytokine, designated interleukin-15 (IL-15), is produced by a wide variety of cells and tissues and shares many biological properties with IL-2. Monoclonal antibodies to the β chain of the IL-2 receptor inhibited the biological activity of IL-15, and IL-15 competed for binding with IL-2, indicating that IL-15 uses components of the IL-2 receptor.

The proliferation and differentiation of T lymphocytes is regulated by cytokines that act in combination with signals induced by the engagement of the T cell antigen receptor. A principal cytokine used by T cells during immune responses is IL-2 (1), itself a product of activated T cells. IL-2 also stimulates a number of other cell types, including B cells, monocytes, lymphokine-activated killer cells, natural killer cells, and glioma cells (2). IL-2 interacts with a specific cell surface receptor (IL-2R) that contains at least three subunits, α , β , and γ (3). A number of other cytokines also stimulate the proliferation of T cells, and recent evidence has suggested that the receptors for several of these cytokines include the γ chain of IL-2R (4). We describe a cytokine whose biological activity resembles that of IL-2 and which also uses components of IL-2R.

In the course of testing supernatants from a simian kidney epithelial cell line, CV-1/EBNA (5), for cytokine activity, it was discovered that these cells produced a soluble factor capable of supporting proliferation of the IL-2-dependent cell line, CTLL (6). The protein responsible for this biological activity was purified from serum-free supernatants of CV-1/EBNA cells by a combination of hydrophobic interaction and anion-exchange chromatography, high-pressure liquid chromatography

(HPLC), and SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (Fig. 1A, bottom). Analysis of the biological activity in the final HPLC fractions (Fig. 1A, top) and in horizontal slices of the SDS-PAGE gel (Fig. 1B) indicated that the activity coincided with a band of approximately 14 to 15 kD. This protein was blotted to a polyvinylidene difluoride (PVDF) membrane, and the NH₂-terminal residues were directly sequenced. We used degenerate oligonucleotide primers on the basis of this amino acid sequence and the polymerase chain reaction (PCR) to clone a 92-base pair (bp) complementary DNA (cDNA) fragment from mRNA of CV-1/EBNA cells. This cloned cDNA fragment was used to probe a plasmid library containing cDNA inserts prepared from mRNA of CV-1/EBNA cells. A full-length cDNA clone was obtained that encodes a 162-amino acid precursor polypeptide containing an unusually long 48-amino acid leader sequence that is cleaved at the experimentally determined NH₂-terminus to form the mature protein. The amino acid sequence (Fig. 1C) exhibits no similarity to any protein in GenBank or EMBL databases. However, IL-15 and IL-2 sequences were compared to determine if there might be structural similarities. The three-dimensional (3D) structure of IL-2 (7) consists of a four-helix bundle, and IL-2 belongs to the helical cytokine family (8). Although the members of this family show no sequence similarity, they show many structural similarities, and IL-15 is no exception. The secondary struc-

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ture prediction for IL-15 shows strong helical moment for regions 1 to 17 and 94 to 112 and supports a four-helix bundle-like structure for this protein. We used the IL-2 structure as a template to build a 3D model of IL-15 with FOLDER, a distance geometry-based homology modeling package (9). The model suggests two disulfide cross-links one of which,

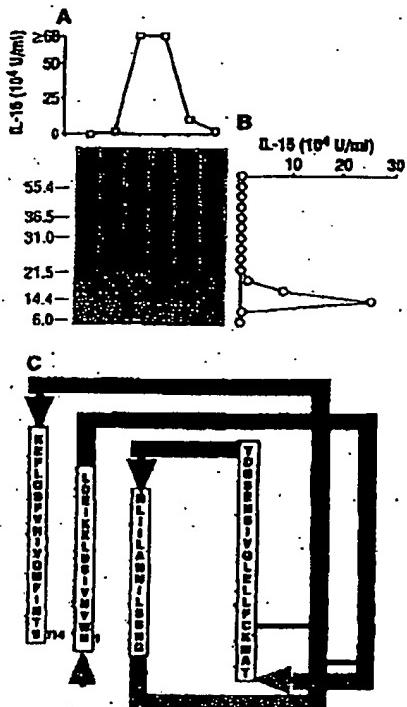


Fig. 1. Purification and cloning of IL-15. (A) (lower panel) Silver-stained gel after SDS-PAGE of active fractions from the final HPLC of the IL-15 purification (17). Molecular sizes are indicated on the left (in kilodaltons). (Upper panel) The activity of each HPLC fraction is graphed directly above the corresponding lane of the gel. (B) The activity eluted from each horizontal slice (of a similar gel of the peak fraction) is depicted alongside the corresponding position on the gel. (C) The deduced amino acid sequence of mature simian IL-15 (18) in a schematic representing its predicted folding topology, with four helices (boxed sequences) in an up-up-down-down configuration, three loops connecting the helices (shaded sequences), and two disulfide crosslinks (darker shading), as suggested by homology modeling of IL-15 with the crystal structure of IL-2 as template with use of the FOLDER program (9). The sequence of simian IL-15 has been submitted to GenBank-EMBL (accession number U03099). Single-letter abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gin; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.

Cys⁴²-Cys⁸⁸, is analogous to the only disulfide in IL-2.

Northern (RNA) blot analysis of a variety of human cell lines identified the IMTLH bone marrow stromal cell line as a source of human IL-15. The simian-derived cDNA was used to probe an IMTLH cDNA library. A single hybridizing clone was isolated that shares 97% sequence identity in the coding region with the simian IL-15 cDNA. The human IL-15 cDNA contains a 316-bp 5' noncoding region preceding an open reading frame of 486 bp and a 400-bp 3' noncoding region.

Expression of IL-15 mRNA was detected by Northern blot analysis of several human tissues. IL-15 mRNA was most abundant in placenta and skeletal muscle (Fig. 2A), with detectable levels in heart, lung, liver, and kidney. The best sources of IL-15 mRNA so far observed have been adherent peripheral blood mononuclear cells (monocyte enriched (PBMCs)) and epithelial and fibroblast cell lines such as CV-1/EBNA and IMTLH. Freshly isolated, uncultured PBMCs (Fig. 2B) also express very low levels of IL-15 mRNA. Activated peripheral blood T cells (PBTs), a rich source of

IL-2 and IFN- γ mRNA, express no detectable IL-15 mRNA, nor do B lymphoblastoid cell lines such as MP-1.

Simian IL-15 was expressed in yeast from a cDNA in which the 5' untranslated sequences and the 48-amino acid leader sequence had been removed. Purified recombinant simian IL-15 stimulated the proliferation of CTLL cells (Fig. 3A) and also phytohemagglutinin (PHA)-activated PBTs (Fig. 3B), in each case to the same extent and with similar potency as IL-2. Both the CD4 $^{+}$ and CD8 $^{+}$ subsets of PBTs responded to IL-15 after activation with PHA (Fig. 3, C and D). IL-15 was also found to stimulate the proliferation of murine antigen-specific T cell clones, including helper and cytotoxic clones (10).

In addition to stimulating the proliferation of CTLL cells and PBTs, IL-15, like IL-2, induces the generation of cytolytic effector cells in vitro (Fig. 4A). The primary in vitro induction of alloantigen-specific cytotoxic T lymphocytes (CTLs) was measured in mixed leukocyte cultures. Non-antigen-specific lymphokine-activated killer (LAK) cells were generated in syngeneic cultures of PBMCs. IL-15 was at

Fig. 2. Northern analysis of human cells and tissues for IL-15 mRNA. Northern blots containing polyadenylated RNAs were hybridized with an antisense probe made by transcription of a human IL-15 cDNA (19). (A) Human tissue blot (Clontech, Palo Alto, California). Molecular sizes are indicated on the left (in kilobases). (B) Blot containing RNA from CV-1/EBNA cells (simian) and human cells including IMTLH cells, a cell line derived from human bone marrow stromal cell culture transformed with pSV3Neo; PBMC, adherent peripheral blood mononuclear cells greatly enriched for monocytes, cultured for 4 hours in lipopolysaccharide; PBT (E-rosetted) stimulated for 4 hours with ionomycin and PMA; PBMC, freshly isolated uncultured peripheral blood mononuclear cells; and MP-1, an EBV-transformed B lymphoblastoid cell line. Cross-hybridization of the IL-15 probe is also seen to 28S ribosomal RNA.

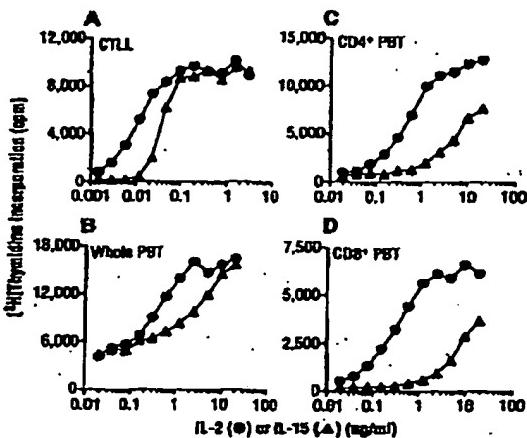
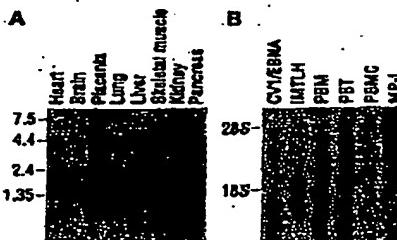


Fig. 3. Regulation of proliferation by IL-15. Purified recombinant simian IL-15 (20) was compared to human recombinant IL-2 (Cetus Oncology Corporation, Emeryville, California), at various concentrations, for induction of proliferation of murine CTLL cells (A) or of PHA-activated human peripheral blood T lymphocytes and their derivative CD4 $^{+}$ and CD8 $^{+}$ subsets (B through D) (15).

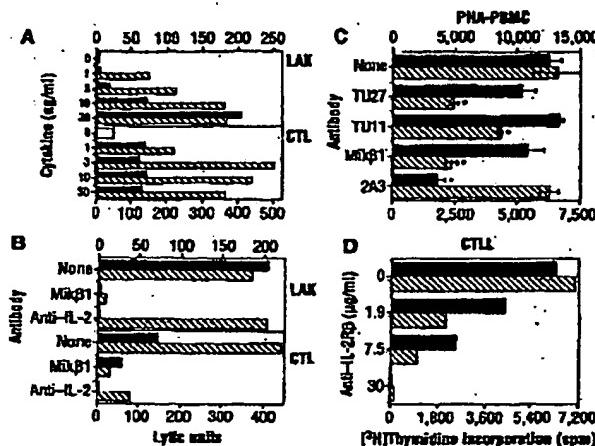
least as potent and effective as IL-2 in these assays.

The similar biological properties of IL-15 and IL-2 suggested that IL-15 might function by inducing the production of IL-2 or by using the same receptor. A neutralizing antibody to IL-2 did not inhibit the induction of LAK cells by IL-15 (Fig. 4B), indicating that IL-15 probably does not function in this assay by inducing IL-2. Furthermore, an antibody to IL-2R α (anti-IL-2R α) (2A3) failed to inhibit IL-15-induced proliferation of PHA-activated

PBMCs (PHA-PBMCs), although this antibody inhibited IL-2-induced proliferation (Fig. 4C). This is further evidence that IL-15 can function in the absence of IL-2. The antibody to IL-2 did, however, inhibit induction of CTLs by IL-15 from resting PBMCs, indicating that under these conditions IL-15 either induces IL-2 or synergizes with the low level of endogenously produced IL-2 to induce functional T cells.

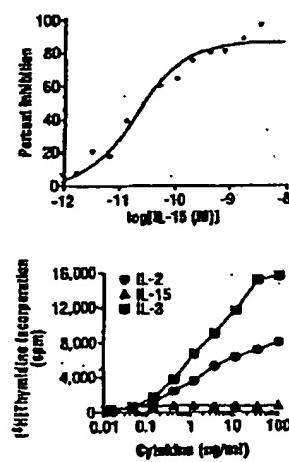
To further compare IL-2 and IL-15, we examined the binding of radiolabeled IL-2 and IL-15 to PHA-activated PBMCs (Fig.

Fig. 4. Inhibition of IL-15 biological activities by antibodies to IL-2R β . (A) Cytolytic activity (lytic units) from human mixed leukocyte cultures (CTL) or syngeneic cultures (LAK) containing various concentrations of cytokine was measured (21) against either the specific allogeneic target cell (CTL) or against the Daudi lymphoblastoid cell line (LAK). (B) Cytolytic activity from cultures parallel to those in (A) containing Mab81 anti-IL-2R β (10 μ g/ml) or sheep anti-IL-2 (100 μ g/ml) in the presence



of either IL-2 or IL-15 (10 ng/ml). (C) Proliferation of PHA-stimulated PBMCs (16) recultured with the indicated antibodies to IL-2R β (each at 10 μ g/ml), Mab81, TU27, and TU11 (22), or to IL-2R α , 2A3 (22). In the presence of IL-2 (top y axis) or IL-15 (bottom y axis) (each at 3 ng/ml). *The mean is different than "none" at the 0.05 level according to Dunnett's procedure for comparing several treatments with a control (23). The data represent the means of four replicate cultures; and the experiment shown is representative of at least five experiments using these and other antibodies to IL-2R β . (D) Proliferation of CTLL cells cultured with various concentrations of anti-IL-2R β (Pharminogen, San Diego, California) in the presence of IL-2 or IL-15 (each at 0.1 ng/ml). The response of the CTLL cells to IL-4 was unaffected by this antibody. Shaded bars, IL-2; hatched bars, IL-15.

Fig. 5. (left) Comparison of 125 I-IL-15 and 125 I-IL-2 binding to PHA-activated PBMCs (24). Scatchard representation of the binding data. **Fig. 6. (top, right)** Inhibition of 125 I-IL-2 binding to YT cells by IL-15. Binding of radiolabeled IL-2 to YT cells (25) was carried out in the presence of increasing concentrations of unlabeled IL-15. Cells were first incubated with the 2A3 antibody to IL-2R α for 60 min, 4°C at 40 μ g/ml. 125 I-IL-2 was used at a concentration of 5×10^{-10} M. **Fig. 7. (bottom, right)** 32D cells respond to IL-2 but not to IL-15. 32D cells were cultured at 50,000 cells per culture in the presence of various concentrations of IL-2 or IL-15.



5). Scatchard analysis indicated that IL-2 binding to these cells shows the well-known two classes of high- ($K_d = 1.48 \times 10^{-11}$ M) and low-affinity receptors, but IL-15 appears to have only a single high-affinity ($K_d = 2.5 \times 10^{-11}$ M) binding site. The number of IL-15 receptors per cell is very similar to the number of high-affinity IL-2 receptors (573 and 426 sites per cell, respectively). To test whether subunits of IL-2R might participate in IL-15 binding, we measured the ability of IL-15 to inhibit binding of radiolabeled IL-2 to receptors on the human YT cell line (11). The YT cell line was chosen because of the low expression of IL-2R α on these cells. IL-15 very effectively competes with IL-2 for binding to YT cells (Fig. 6), suggesting that IL-2 and IL-15 share common binding sites on these cells. IL-1 and IL-4, both of which bind to their own sites on YT cells, failed to compete for IL-2 binding. Experiments with blocking monoclonal antibodies confirmed the participation of IL-2R chains in IL-15 binding and function. Whereas the anti-IL-2R α did not inhibit IL-15-induced proliferation of T cells, antibodies to IL-2R β , such as TU27, have been shown to neutralize IL-2-dependent T cell proliferation (such as in Fig. 4C) only when combined with anti-IL-2R α (12). These antibodies to IL-2R β consistently inhibit IL-15 more effectively than they inhibit IL-2. In fact, even antibodies like TU11, which do not inhibit IL-2 under any circumstances (13), will inhibit IL-15, suggesting that IL-15 binds to IL-2R β differently than does IL-2. These data confirm that stimulation by IL-15 requires interaction of IL-15 with components of IL-2R, including IL-2R β and probably IL-2R γ , but not IL-2R α .

Although some activities are shared by IL-2 and IL-15, there are also differences between the biological effects of the two cytokines. For example, the IL-3-dependent cell line, 32D (14), expresses the complete IL-2R and proliferates vigorously in response to IL-2, but responded poorly or not at all to IL-15 (Fig. 7). These data suggest that IL-15 may use another receptor component not shared by IL-2R and not expressed by 32D cells. Thus, there are likely to be biological activities of IL-15 not shared by IL-2.

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15. PBMCs were selected from fresh PBMCs by E-rosette formation, and CD4+ and CD8+ subsets were further isolated by antibody affinity to paramagnetic microspheres with magnetic cell sorter (MACS, Miltenyi Biotech, Sunnyvale, CA). T cells were activated for 72 hours with PHA followed by 24 hours in IL-2-containing medium. T cell blasts were then harvested, washed, and used. Test cultures contained 50,000 T cells per culture or 2000 CTL cells per culture. Culture medium was supplemented as described [K. Grabstein et al., *J. Exp. Med.* 163, 1405 (1986)], and 0.5 pCi of [³H]thymidine was added for the last 4 hours of culture. Cells were collected onto glass fiber filters and radioactivity was determined by a sealed-gas ionization.
16. PBMCs, prepared from fresh whole blood by Ficoll Hypaque density gradient centrifugation, were activated by culture with PHA as described above for PBTCs (15).
17. IL-15 was purified from 64 liters of supernatant of CV-1/EBNA cells by ultracentrifugation (YM-30), hydrophobic chromatography (Phenyl Sepharose CL-4B), anion-exchange chromatography (DEAE Sephadex and Mono Q fast protein liquid chromatography), reversed-phase HPLC (C4, 5 μm) eluted with an acetonitrile gradient in 0.1% trifluoroacetic acid (TFA) [D. L. Urda et al., *J. Chromatogr.* 258, 171 (1984)], reversed-phase HPLC eluted with an n-propanol gradient in TFA, and SDS-PAGE.
18. Proteins were electroblotted from the SDS gel to a PVDF membrane. The protein band corresponding to the IL-15 activity was cut out, and the sequence of the 33 NH₂-terminal residues was determined by Edman degradation. Two degenerate oligonucleotide mixtures encoding all possible codon usages of residues 1 to 8 and the complement of all possible codon usages of residues 26 to 31 (omitting position 3 of Val²⁷) were synthesized. First-strand cDNA synthesized from CV-1/EBNA mRNA was amplified by PCR with the oligonucleotide mixtures as primers. This yielded a 82-kbp DNA fragment that was cloned into pBluescript SK+. A hybridization probe prepared from this DNA fragment was used to isolate a cDNA clone containing the complete IL-15 coding region from a cDNA library constructed from CV-1/EBNA mRNA essentially as described [D. M. Anderson et al., *Cel.* 63, 235 (1990)].
19. A simian IL-15 probe was prepared by labeling of the purified simian IL-15 cDNA with random primers. Northern blot analysis with this probe identified the human IMTLH bone marrow-derived stromal cell line as a source of human IL-15 mRNA. Southern blots of pools of an IMTLH cDNA library were probed to identify a positive pool and subsequently to isolate a human IL-15 cDNA.
20. A PCR-generated DNA fragment, containing the simian IL-15 coding region minus the 48-amino acid leader sequence, was ligated into a yeast expression vector that directs secretion of the recombinant protein into the yeast medium [V. Price et al., *Gene* 55, 287 (1987)]. Recombinant IL-15 was purified from the yeast supernatant as described above for the CV-1-derived IL-15 protein, excluding ultracentrifugation and ion exchange. The purity and concentration of IL-15 were confirmed by amino acid analysis.
21. Human PBMCs from one donor (45×10^6 per culture) were cultured with irradiated PBMCs (5×10^6 per culture) from either an allogeneic donor (CTL) or from the autologous donor (LAk) in cultures containing various concentrations of either IL-2 or IL-15, or no cytokine. Cultures were done as described [M. B. Widmer et al., *J. Exp. Med.* 163, 1447 (1987)] and harvested after 6 days (LAk) or 7 days (CTL) and assayed for cytolytic activity against ⁵¹Cr-labeled targets. The lytic assay contained variable numbers of the responding peripheral blood lymphocytes cultured with 1000 labeled targets in 200 μl of medium in V-bottomed wells, and supernatants were collected after 4 hours of incubation. Lytic units were calculated as the inverse of the fraction of the responding culture required to generate 50% (CTL) or 30% (LAk) of the maximum specific ⁵¹Cr release.
22. Mab1 was purchased from Nichirei Corp., Tokyo, Japan; TU11 and TU27 were provided by K. Sugamura, Sendai, Japan; 2A3 was produced at Immuno.
23. C. W. Dunnett, *J. Am. Stat. Assoc.* 50, 1083 (1955).
24. Activated PBMCs (16) were incubated for 2 hours in medium without PMA or growth factors. IL-15 and IL-2 binding were carried out at 4°C for 60 min in RPMI 1640 containing 5% bovine serum albumin and 0.15% NaNO₂. IL-2 and IL-15 were radioiodinated as described [J. Park et al., *J. Biol. Chem.* 261, 4177 (1986)] and residual biological activity. Preliminary experiments established that equilibrium binding was attained under these conditions (J. Giri and M. Andrich, personal communication).
25. YT cells used are a subclone of the human NK-cell YT cell line and were provided by M. Calligari, Research Park Memorial Institute, Buffalo, NY.
26. We thank S. D. Lupton and R. J. Tushchield for the IMTLH cell line, M. R. Comissi and D. P. Geisinger for the IMTLH cDNA library, T. Hollingsworth for DNA sequencing analysis, C. J. March and M. Gerhart for protein sequencing analysis, R. Jerry for the cDNA cloning plasmid, J. King for the yeast expression construct, T. W. Tough and L. Erickson for technical assistance, and M. B. Widmer and M. K. Spragg for reviewing the manuscript.

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Enhancer Point Mutation Results in a Homeotic Transformation in *Drosophila*

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Michael B. O'Connor*

In *Drosophila*, the misexpression or altered activity of genes from the bithorax complex results in homeotic transformations. One of these genes, *abd-A*, normally specifies the identity of the second through fourth abdominal segments (A2 to A4). In the dominant *Hyparabdominal* mutations (*Hab*), portions of the third thoracic segment (T3) are transformed toward A2 as the result of ectopic *abd-A* expression. Sequence analysis and deoxyribonuclease I footprinting demonstrate that the misexpression of *abd-A* in two independent *Hab* mutations results from the same single base change in a binding site for the gap gene Krüppel protein. These results establish that the spatial limits of the homeotic genes are directly regulated by gap gene products.

The establishment of correct segmental identity in *Drosophila melanogaster* requires the proper function and expression of genes located in the antenapedia and bithorax complexes (reviewed in (1–3)). In the bithorax complex, loss-of-function mutations typically result in transformations of posterior segments toward more anterior fates, whereas the ectopic activation of homeotic genes along the anterior-posterior axis produces dominant, gain-of-function phenotypes in which anterior segments are transformed toward more posterior identities (4–8). The initial activation of homeotic gene expression appears to be regulated by the segmentation

gene products (9–15). For example, mutations in the gap gene *hunchback* (*hb*) result in an anterior shift of *Ubx* expression (12), whereas mutations in *Krüppel*, *trip*, and *giant* cause ectopic activation of *Abd-B* (13–16). In several cases, incomplete homeotic regulatory elements containing *hb* binding sites have been shown to confer spatially restricted patterns of gene expression when positioned next to a *LacZ* reporter gene (9–11). However, as a result of the large size of homeotic regulatory regions (50 to 100 kb), the precise roles of these individual elements within the context of a complete regulatory domain has remained elusive.

We have studied the *Hab-1* and *Hab-2* mutations, two gain-of-function alleles that ectopically express the *abd-A* protein (ABD-A). The *Hab-1* and *Hab-2* alleles were discovered by E. B. Lewis and I. Duncan, respectively, and Lewis has proposed that they likely affect a homeotic regulatory element (4, 15). The *Hab* mutations cause dominant transformations of portions of T3 toward A2 as a result of

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IL-21 Limits NK Cell Responses and Promotes Antigen-Specific T Cell Activation: A Mediator of the Transition from Innate to Adaptive Immunity

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Summary

IFN α/β , IL-12, and IL-15 regulate NK cell activation and expansion, but signals triggering resolution of the NK response upon induction of adaptive immunity remain to be defined. We now report that IL-21, a product of activated T cells, may serve this function. Mice lacking IL-21R ($IL-21R^{-/-}$) had normal NK cell development but no detectable responses to IL-21. IL-21 enhanced cytotoxic activity and IFN γ production by activated murine NK cells but did not support their viability, thus limiting their duration of activation. Furthermore, IL-21 blocked IL-15-induced expansion of resting NK cells, thus preventing the initiation of further innate responses. In contrast, IL-21 enhanced the proliferation, IFN γ production, and cytotoxic function of CD8 $+$ effector T cells in an allogeneic MLR. These observations suggest that IL-21 promotes the transition between innate and adaptive immunity.

Introduction

Innate immune mechanisms shape the adaptive cellular responses that follow. In turn, adaptive immunity likely feeds back to limit ongoing innate responses, but the mechanisms by which this occurs are poorly understood. During acute pathogen infections, the NK cell response begins within hours, as IFN α/β , IL-12, IL-15, and IL-18 generated by infected cells stimulate NK cytotoxicity, cytokine production, and expansion (Biron et al., 1999). Along with enhanced effector function, the maturation of NK cells is ultimately accompanied by their terminal differentiation. An emerging view (Loza and Perussia, 2001) supports a sequential process of NK cell development in the human system resulting in generation of committed IFN γ -producing effector NK cells whose subsequent terminal differentiation coincides with abatement of the innate response. Although cytokine regulation of initial NK cell recruitment and activation has been intensively studied (Biron et al., 1999), the signals responsible for resolution of this re-

sponse remain to be defined. The concordance of decreased NK cell responses with the emergence of antigen-specific T cells makes it likely that T cell-derived factors influence the final steps of NK cell maturation.

IL-21 is a newly described cytokine produced by activated T cells with effects that include costimulation of T cell proliferation, potentiation of NK cell maturation from bone marrow progenitors, and activation of peripheral NK cells in human assay systems (Parrish-Novak et al., 2000). The cytokine itself is related to IL-2, IL-4, and IL-15, and cellular effects are mediated through a class I cytokine family receptor, IL-21R (Parrish-Novak et al., 2000; Ozaki et al., 2000). IL-21R has homology to the shared β chain of the IL-2 and IL-15 receptors (Ozaki et al., 2000), interacts with the common γ cytokine receptor chain (γc) (Asao et al., 2001), and appears to signal by association with Jak1 (Ozaki et al., 2000; Asao et al., 2001) and Jak3 (Asao et al., 2001), and phosphorylation of STAT1 and STAT3 (Asao et al., 2001) or STAT5 (Ozaki et al., 2000). Although signaling chains other than γc have not been detected, the many functions of IL-21 so far described, the widespread lymphoid distribution of its receptor (Parrish-Novak et al., 2000), and indications that coexpression of IL-21R and γc , while necessary, may not be sufficient to mediate signaling in all cell types (Ozaki et al., 2000) raise the potential for additional receptor forms.

To explore the role of IL-21 in regulating innate and adaptive immunity, two complementary approaches were employed. *In vitro* studies examined effects of IL-21 on NK and T lymphocyte growth and function, alone and in conjunction with IL-15. Reciprocal studies done with mice lacking functional IL-21R ($IL-21R^{-/-}$) addressed the influence of a lack of IL-21 signaling on innate and adaptive immunity. $IL-21R^{-/-}$ mice had normal lymphocyte compartments and no NK cell deficiency, an unexpected finding given the previously described role of IL-21 in human NK cell maturation (Parrish-Novak et al., 2000). Cells from these mice did not display any response to IL-21 detectable in these assays, including effects on T cell proliferation, NK cell activation and expansion, and cytokine receptor expression. Our findings in the mouse reveal that innate NK cell responses and the cytokine-driven TCR-independent outgrowth of CD44 hi CD8 $^{+}$ T cells were antagonized by IL-21, whereas antigen-driven T cell activation in an allogeneic MLR was stimulated. As a product of activated T lymphocytes that acts to limit ongoing NK cell expansion while promoting antigen-specific T cell-mediated immunity, IL-21 may be a key element in the transition between innate and adaptive immune responses.

Results

Generation and Characterization of $IL-21R^{-/-}$ Mice
Mice were made genetically deficient in IL-21R ($IL-21R^{-/-}$) as described in Experimental Procedures and outlined in Figure 1A. $IL-21R^{-/-}$ mice were viable and fertile and were bred on both BALB/c × C57BL/6

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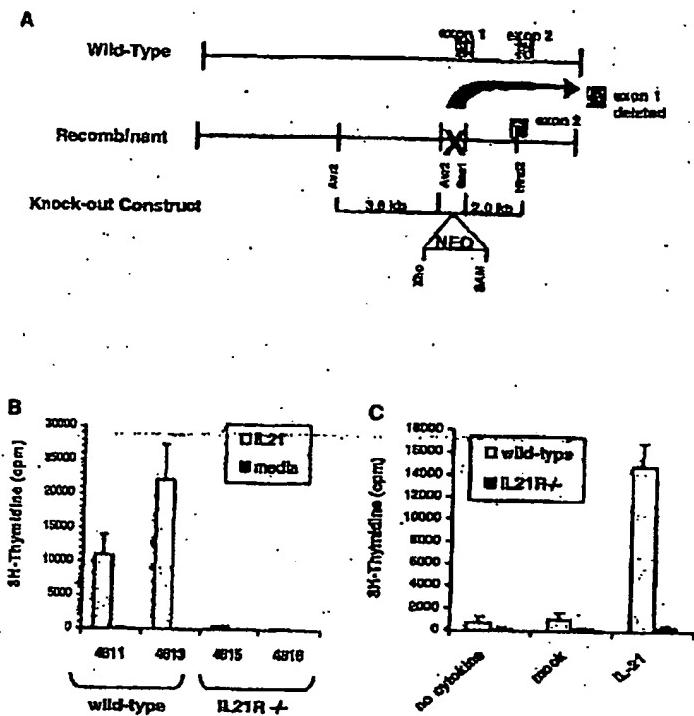


Figure 1. Functional inactivation of the IL-21R Gene

(A) The structure of the wild-type allele, recombinant gene, and knockout construct are shown at top, middle, and bottom, respectively. The knockout construct, consisting of a neomycin resistance cassette flanked by appropriate linkers for homologous recombination, was targeted to replace the IL-21R exon 1 sequence.

(B and C) Thymocytes isolated from (B) wild-type (4611 and 4613) or IL-21R^{-/-} (4615 and 4616) mice or (C) lymph node cells pooled from wild-type or IL-21R^{-/-} mice were incubated 3 days with media (no cytokine), IL-21 (30 U/ml), or COS mock control on anti-CD3-coated plates, and [³H]thymidine incorporation was assayed over the final 5 hr.

and C57BL/6 backgrounds. Adult IL-21R^{-/-} mice had normal numbers of peripheral blood erythrocytes, monocytes, granulocytes, and lymphocytes (data not shown). Phenotypic analysis of T cell, B cell, and monocyte populations in spleen, lymph node, and thymus showed no significant differences between IL-21R^{-/-} and wild-type (data not shown). In the serum, IL-21R^{-/-} mice were found to have approximately 3-fold lower levels of IgG1 ($p < 0.05$), 2-fold lower levels of IgG2b ($p < 0.05$), and 3-fold higher levels of IgE ($p < 0.02$) as compared to wild-type mice (data not shown).

Because reagents are not currently available for detecting cell surface expression of murine IL-21R by phenotypic analysis, the absence of functional receptor was confirmed by lack of IL-21 responsiveness in cells isolated from IL-21R^{-/-} mice. In accordance with the observations of Parrish-Novak et al. (2000), IL-21 enhanced the proliferation of thymocytes from wild-type but not IL-21R^{-/-} mice in response to suboptimal concentrations of anti-CD3 (Figure 1B). In addition, IL-21 was found to enhance anti-CD3-responsiveness of lymph node T cells from wild-type but not IL-21R^{-/-} mice (Figure 1C). These observations support the functional inactivation of the IL-21R gene in IL-21R^{-/-} mice.

IL-21R^{-/-} Mice Have Normal NK Cell Numbers and Display Full NK Activation In Vivo and In Vitro

Parrish-Novak et al. (2000) observed that IL-21 enhanced the generation of NK cells from human bone marrow progenitors. To determine if a lack of IL-21R

affected the generation of mature NK cells, these cells were quantitated in spleens of IL-21R^{-/-} mice. Results with mice on BALB/c × C57BL/6 and C57BL/6 backgrounds were indistinguishable. Both the percentages (Figure 2A) and the total numbers of NK cells ($3.08 \pm 0.78 \times 10^6$ /spleen for wild-type and $3.77 \pm 0.91 \times 10^6$ /spleen for IL-21R^{-/-} mice) were equivalent, indicating that IL-21R^{-/-} mice had no intrinsic defect in the generation of phenotypically mature NK cells.

Having established that IL-21R^{-/-} mice have a full complement of spleen NK cells, we next examined the ability of these cells to undergo activation *in vivo* and *in vitro*. All functional studies were done with mice on the C57BL/6 background, unless otherwise noted. NK cells from IL-21R^{-/-} mice were fully able to respond to poly I:C *in vivo* (Figure 2B) or IL-15 *in vitro* (Figure 2C) with induction of lytic activity that was indistinguishable from that found in NK cells from wild-type animals. This indicates that NK cells from IL-21R^{-/-} mice are fully responsive to typical activating agents *in vivo* and *in vitro*.

IL-21 Reduces IL-15-Mediated Expansion but Has No Effect on Activation of Resting NK Cells

In addition to enhancing effector function, IL-15 enhances NK cell survival and proliferation (Carson et al., 1997), and these effects were comparable using splenic NK cells of wild-type and IL-21R^{-/-} mice (Figure 3A). IL-21 alone did not support expansion of NK cells *in vitro* (data not shown). Therefore, to study the effects of IL-21 on NK cell outgrowth, IL-21 was used in conjunc-

Activation of NK and T Cells from IL-21R^{-/-} Mice
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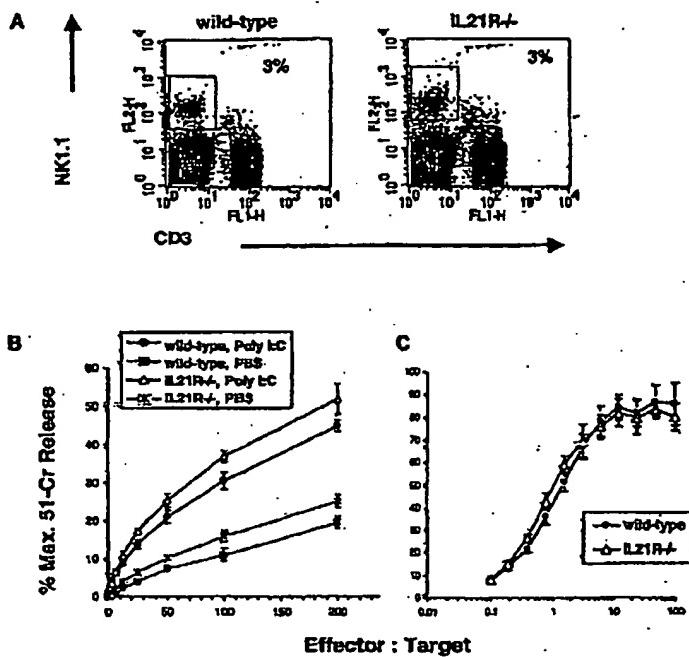


Figure 2. IL-21R^{-/-} Mice Have Normal NK Cell Number and Display Full NK Cell Activation In Vivo and In Vitro

(A) Flow cytometric analysis of spleen lymphocytes from wild-type or IL-21R^{-/-} mice. NK cells, identified as NK1.1⁺/CD3⁻, are boxed.

(B) Wild-type or IL-21R^{-/-} mice were injected i.p. with poly I:C or PBS control, and spleens were harvested 1.5 days later.

(C) Spleen cells isolated from wild-type or IL-21R^{-/-} mice were treated in vitro with IL-15 (50 ng/ml) for 7 days, then used as effector in a 5 hr 51-Cr release assay against YAC-1 targets. Cells were pooled from 2-3 mice per group.

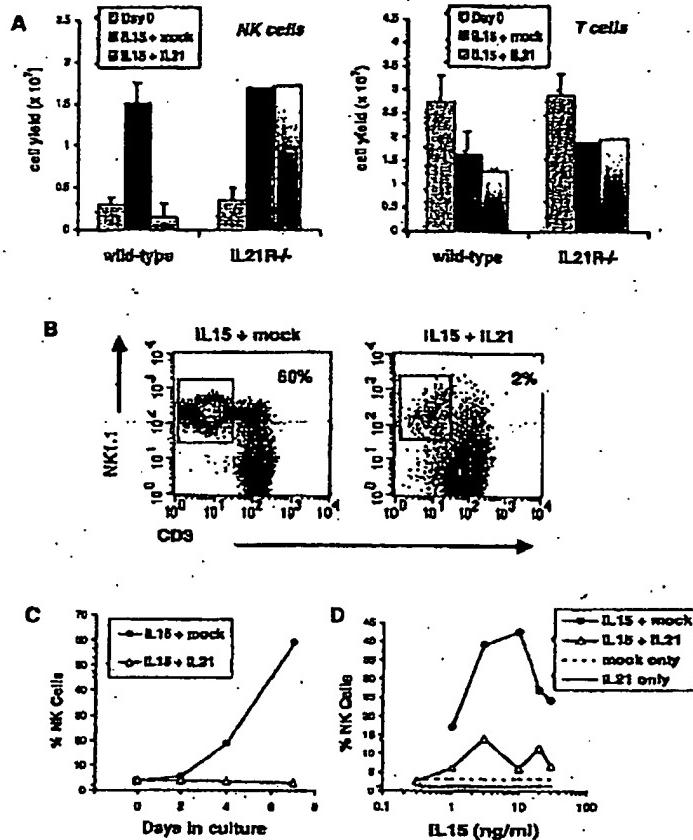
tion with IL-15. For wild-type but not IL-21R^{-/-} cells, addition of IL-21 inhibited IL-15-mediated NK cell expansion in a 7-day culture (Figures 3A and 3B) but had no effect on total T cell numbers, which dropped ~30% in these cytokine-driven, antigen-independent cultures (Figure 3A). Similar findings were seen with IL-2-expanded cultures (data not shown). Kinetic analysis revealed that IL-21 blocked IL-15-mediated NK cell proliferation throughout the culture period (Figure 3C). Rather than shifting the effective dose of IL-15 required for NK cell expansion, IL-21 blocked NK cell outgrowth over the entire range of IL-15 concentrations to which the NK cells responded (Figure 3D). Thus, IL-21 limits outgrowth of NK cells in response to IL-15.

To further examine IL-21 effects on NK cell activation, freshly isolated murine splenocytes were cultured for 2-3 days in the presence of IL-21 and/or IL-15 and tested for cytotoxicity against NK-sensitive YAC-1 target cells. In response to IL-15, resting NK cells from both wild-type and IL-21R^{-/-} mice became actively cytolytic (Figures 2C, 4A, and 4D). In contrast, IL-21 did not promote activation of resting NK cells (Figures 4A and 4D) and had no effect on cytolytic potential per cell induced by IL-15 (Figure 4A), although absolute NK cell numbers were greatly reduced in cultures containing IL-15 + IL-21 (Figure 3A). Taken together, these results indicate that IL-21 antagonizes IL-15-induced growth but not activation of resting NK cells.

IL-21 Enhances Cytotoxicity of Previously Activated NK Cells and Induces Their Apoptosis
Parish-Novak et al. (2000) found that IL-21 stimulates cytotoxicity of human NK cells enriched by positive selection from peripheral blood. Our results in the mouse

appeared contradictory, as no activation of murine splenic NK cells was seen in response to IL-21 (Figure 4A). In an attempt to reconcile these observations, we reasoned that human NK cells, continuously challenged with environmental agents, may exist in a heightened state of activation as compared to NK cells of a mouse residing in a specific pathogen-free facility. Therefore, IL-21 effects were examined on NK cells from mice that had been challenged in vivo with poly I:C to induce their activation. Cells harvested from mice treated with poly I:C or PBS control were restimulated for 2-3 days in vitro with IL-15, IL-21, or COS mock control, then assayed for lytic activity. In contrast to its effects on NK cells from resting mice, IL-21 alone induced a high level of cytotoxicity in NK cells from poly I:C-treated mice (Figure 4B). In order to determine whether heightened IL-21 responsiveness would also follow NK cell activation in vitro, splenocytes were cultured for 7 days with IL-15, then restimulated for 2 days with IL-21, IL-15, or the combination. In this case, restimulation with either IL-21 or IL-15 alone greatly enhanced NK cytolytic function (Figure 4C). Results shown in Figures 4B and 4C and other experiments (data not shown) suggest an additive effect of IL-15 and IL-21 on NK cell activation, with no indication of synergy. Cells from IL-21R^{-/-} mice displayed full cytolytic activation with IL-15 but did not respond to IL-21 (Figures 4D-4F). For these cells, IL-15 + IL-21 produced no greater activation than IL-15 alone (Figure 4F).

In addition to mediating cytotoxicity, activated NK cells produce IFN γ in an IL-12-dependent manner. In order to determine whether IL-21 treatment of activated NK cells affected IFN γ production, spleen cells stimulated in vitro for 7 days with IL-15 were rechallenged for



2 days with IL-15 and/or IL-21. Treatment of activated NK cells with IL-21 greatly enhanced IL-12-driven IFN γ production, and the response was further potentiated by the combination of IL-15 and IL-21 (Figure 5A). In

addition to boosting IL-12-dependent IFN γ production, IL-21 treatment also resulted in high levels of IFN γ production in the absence of added IL-12 (Figure 5A). In contrast, when cells from IL-21R^{-/-} mice were activated

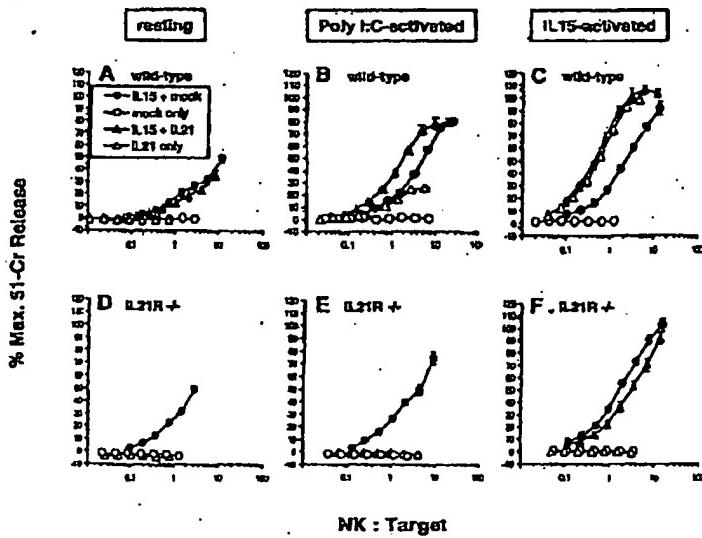


Figure 4. IL-21 Boosts NK Cytotoxicity in Spleen Cells Activated with Poly I:C In Vivo or IL-15 In Vitro but Does Not Induce Activation of Resting NK Cells

Resting cells: spleen cells isolated from wild-type (A) or IL-21R^{-/-} (D) mice were treated for 2–3 days with IL-15 (10 ng/ml) + COS mock control (●), COS mock control only (○), IL-21 (12.6 U/ml) only (▲), or (A) IL-15 + IL-21 (▲). Poly I:C-activated cells: spleen cells isolated from wild-type (B) or IL-21R^{-/-} (E) mice 1.5 days post I.p. administration of poly I:C were cultured 2 days with the indicated treatment. IL-15-activated cells: spleen cells isolated from untreated wild-type (C) or IL-21R^{-/-} (F) mice were cultured 7 days with IL-15 (10 ng/ml), then washed and restimulated for 2 days with the treatments as described above. Data are shown as mean \pm SD of replicate wells in a 5 hr 51-Cr release assay against YAC-1 targets. Effector:target ratios were corrected for the percentage of NK1.1⁺/CD3⁻ cells in each culture, identified by flow cytometry.

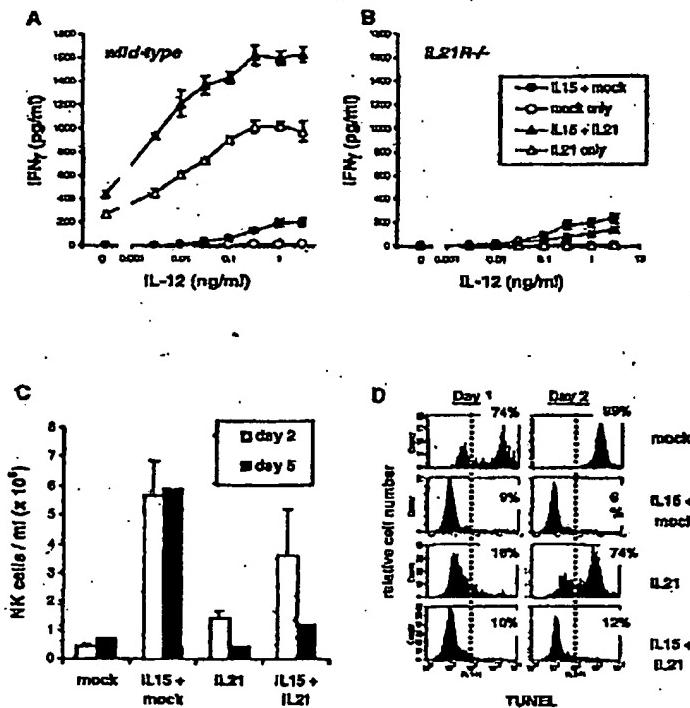


Figure 5. IL-21 Boosts IFN γ Production by IL-15-Activated Spleen Cells but Blocks Their Growth

(A and B) Spleen cells isolated from wild-type (A) or IL-21R^{-/-} (B) mice were cultured 7 days with IL-15 (10 ng/ml), then restimulated for 2 days with IL-15 (10 ng/ml) + COS mock control (●), COS mock control only (○), IL-15 (10 ng/ml) + IL-21 (12.5 U/ml) (▲), or IL-21 (12.5 U/ml) only (△). For determination of IFN γ production, cells were washed free of cytokine and challenged for 24 hr with the indicated concentration of murine IL-12. Data are shown as mean \pm SD of IFN γ levels in replicate culture wells.

(C) Cell concentrations from cultures activated 7 days with IL-15 and restimulated 2 or 5 days with the indicated treatment at the doses indicated above. The NK cell concentration on day 7 of culture with IL-15 (prior to restimulation) was 4.1×10^6 /ml.

(D) Apoptosis in spleen cell cultures expanded for 5 days with IL-15 (10 ng/ml) and restimulated for 1 or 2 days with agents as described above. At each time point, cells were stained for surface expression of NK1.1 and CD3 and intracellular TUNEL. Data are shown for NK cells gated as NK1.1 $^+$ /CD3 $^+$.

with IL-15, then challenged with IL-21, no enhanced spontaneous or IL-12-driven IFN γ production was found (Figure 5B).

Thus, previously stimulated but not resting NK cells showed strong induction of cytolytic activity (Figures 4A–4C) and IFN γ production (Figure 5A) when exposed to IL-21. Experiments using FACS-sorted populations of >95% pure NK and T cells confirmed that both activities could be attributed almost exclusively to NK cells in these cultures (data not shown). Interestingly, however, enhanced effector responses were not accompanied by growth effects. Cultures of IL-15-stimulated NK cells that were rechallenged for 2 days with IL-21 contained fewer NK cells than those maintained in IL-15 (Figure 5C). Examination of these cultures after 5 days of challenge confirmed that IL-21 not only failed to sustain NK viability but, when used in combination with IL-15, IL-21 reduced NK cell survival mediated by that cytokine (Figure 5C). Thus, IL-21 boosted the effector functions of activated NK cells but did not promote their viability, such that although activity per cell was increased, their number was sharply reduced.

Because of its effects on viability, we examined the influence of IL-21 on NK cell death by apoptosis triggered by removal of IL-15. The TUNEL staining method was used on IL-15-expanded cultures restimulated for 2 days with IL-15, IL-21, or COS mock control supernatant. In cultures treated with COS mock control, most NK cells were apoptotic within 1 day, indicating that apoptosis occurs rapidly upon withdrawal of IL-15. As compared to COS mock control, IL-21 delayed the apoptosis caused by removal of IL-15. Nevertheless, after

2 days of restimulation with IL-21 the majority of NK cells in the culture were apoptotic (Figure 5D). Taken together with observations that cells in similarly treated cultures restimulated for 2 days with IL-21 displayed high levels of cytolytic activity (Figure 4C) and IFN γ production (Figure 5A), these findings indicate that IL-21 induces high levels of effector function in NK cells undergoing apoptosis. Restimulation with IL-15 + IL-21 also resulted in enhanced NK cell effector function (Figures 4C and 5A) but prevented or delayed apoptosis (Figure 5D). This indicates that apoptosis is not a necessary correlate of the IL-21-mediated enhancement of NK cell effector function.

IL-21 Blocks IL-15-Dependent Expansion of CD44 $^+$ CD8 $^+$ TCR-Independent T Cells and T Cell Cytokine Receptor Expression

In the mouse, IL-15 in the absence of a TCR signal induces proliferation of CD8 $^+$ T cells expressing high levels of CD44, corresponding to a "memory" phenotype (Zhang et al., 1998; Sprent and Surh, 2001). In accordance with this, IL-15-expanded spleen CD8 $^+$ T cells from either wild-type or IL-21R^{-/-} mice were skewed toward high-level expression of CD44 (Figure 6A). Addition of IL-21 counteracted the expansion of CD44 $^+$ CD8 $^+$ T cells from wild-type mice but had no effect on cells from IL-21R^{-/-} mice (Figure 6A). Because TCR-Independent CD44 $^+$ CD8 $^+$ T cells are responsive to IFN γ in addition to IL-15 (Tough et al., 2001), expression of the IFN γ receptor, CD119, was also examined. IL-21 also prevented the expansion of cells expressing this marker in

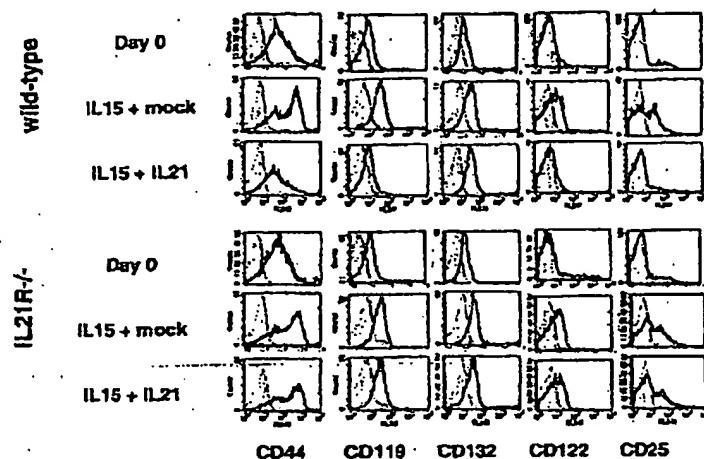
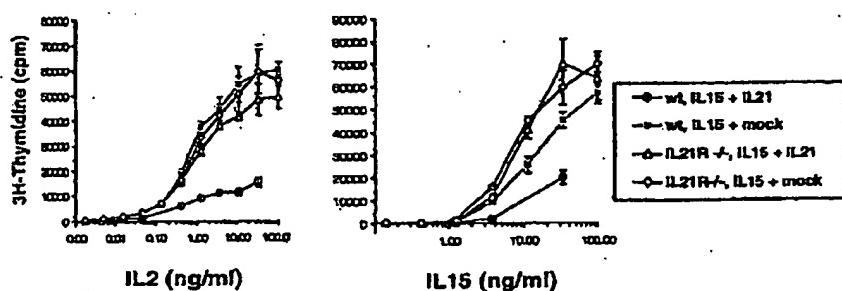
A**B**

Figure 6. IL-21 Prevents IL-15-Induced Expansion of CD44⁺ CD8⁺ T Cells

Spleen cells isolated from IL-21R^{-/-} or wild-type mice (BALB/c × C57BL/6 background) were cultured 7 days in IL-15 (50 ng/ml) + COS mock supernatant or IL-15 + IL-21 (25 U/ml).

(A) Cell surface marker expression was analyzed on day 0 and on day 7 of culture. CD44, CD119, and CD132 were analyzed on gated CD8⁺ CD44⁺ cells. CD122 and CD25 were analyzed on total CD3⁺ cells. Appropriate gates were established using three-color flow cytometry.

(B) Spleen cells grown 7 days with IL-15 + COS mock supernatant or IL-15 + IL-21 were washed free of cytokine, then replated with the indicated concentration of IL-2 or IL-15. [³H]thymidine incorporation was assayed over 24 hr.

response to IL-15 on cells from wild-type but not IL-21R^{-/-} mice (Figure 6A).

To further examine IL-21 effects on the cytokine responsiveness of T cells expanded with IL-15, we examined CD25 (IL-2R α), CD122 (shared β chain of IL-2R and IL-15R), and CD132 (γ c) levels on spleen T cells following exposure to IL-15 in the presence or absence of IL-21. Both CD25 and CD122 expression was increased upon IL-15 treatment of cells from wild-type and IL-21R^{-/-} mice. Addition of IL-21 prevented this receptor induction on T cells from wild-type mice but had no effect on cells from IL-21R^{-/-} mice (Figure 6A). Expression of γ c (CD132) was not affected by IL-21 (Figure 6A). The decreased expression of receptor chains suggested that in the presence of IL-21 the responsiveness of splenic T cells to IL-2 or IL-15 would

be reduced. In accordance with this, wild-type spleen cells that had been expanded with IL-15 in the presence of IL-21 showed less proliferation in response to IL-2 or IL-15 than those maintained in the absence of IL-21. Cells from IL-21R^{-/-} mice were unaffected by IL-21 (Figure 6B). Thus, IL-21 prevented IL-15-driven, antigen-independent T cell responses, including the expansion of CD44⁺ CD8⁺ cells and the increased expression of functional cytokine receptors.

IL-21 Enhances T Cell Responses to Alloantigen

Having shown that IL-21 blocks the cytokine-driven expansion of NK cells and of antigen-independent CD44⁺ CD8⁺ T cells, we asked whether IL-21 would have similar effects in an antigen-driven T cell response, using a mixed lymphocyte reaction system. Purified

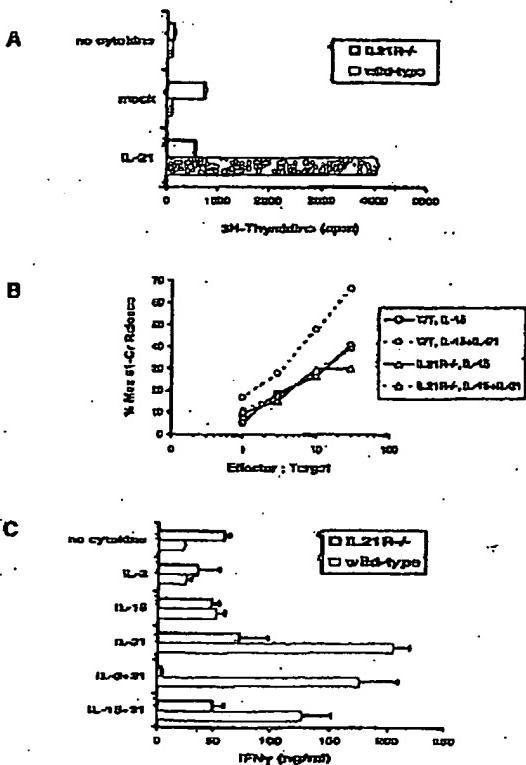


Figure 7. IL-21 Enhances T Cell Proliferation and Activation in Response to Alloantigen
(A) T cell enriched populations from lymph nodes of wild-type and IL-21R^{-/-} mice (BALB/c × C57BL/6 background) were cultured 3–4 days with irradiated allogeneic spleen cells in the presence of no cytokine, IL-21 (10 U/ml), or COS mock control. [H]thymidine incorporation was assayed over the final 12 hr of culture.
(B) Cells isolated from cultures of primary alloantigenic stimulation were assayed for cytotoxicity against a B6genomic target cells in a 4 hr 51-Cr release assay. Data are corrected for percentage of CD3⁺ T cells under each priming condition.
(C) IFNγ production was assayed from T cells "primed" as indicated following a 40 hr secondary stimulation with fresh allogeneic spleen cells and no added cytokine.

Lymph node T cells from wild-type or IL-21R^{-/-} (H-2^b) mice were activated for 3–5 days with irradiated allogeneic splenocytes (H-2^a) in the presence of IL-21 or control supernatant. Similar to results with anti-CD3 stimulation (Figure 1C), IL-21 enhanced alloantigen stimulation of wild-type but not IL-21R^{-/-} T cells (Figure 7A). T cells from both IL-21R^{-/-} and wild-type mice exhibited enhanced proliferation to alloantigen in the presence of IL-2 or IL-15 (data not shown) and thus have no intrinsic defects in responsiveness. Stimulation of T cells results in the development of effector functions, including CTL activity and IFNγ production. Therefore, we compared the ability of IL-21R^{-/-} and wild-type T cells to differentiate into allospecific effectors in response to IL-21 and related cytokines. For wild-type cells, IL-21 alone primed the development of allospecific CTL activity, similar to

IL-2, IL-15, or IL-12 (data not shown), but was not effective with IL-21R^{-/-} cells. Therefore, IL-15 or IL-2 was added to generate sufficient numbers of cells to perform these assays. T cells from wild-type or IL-21R^{-/-} mice primed with alloantigen and IL-15 displayed strong CTL activity toward allospecific target cells (Figure 7B). Priming in the presence of IL-15 + IL-21 further enhanced the development of lytic activity in wild-type but not IL-21R^{-/-} cultures, indicating that IL-15 and IL-21 cooperatively enhance CTL differentiation. Similar results were observed when cells were primed in the presence of IL-2 (data not shown). After priming with alloantigen APCs and the indicated cytokines, wild-type or IL-21R^{-/-} T cells were restimulated, and IFNγ production was determined as another measure of effector function. Wild-type T cells primed in the presence of IL-21, alone or in combination with IL-2 or IL-15, secreted higher levels of IFNγ compared to those primed with IL-2 or IL-15 alone (Figure 7C). Taken together, these results suggest that IL-21 enhances in vitro T cell responses to alloantigen in primary stimulation and results in the generation of more potent effector T cells.

Discussion

In this study, we have defined a role for IL-21 in the maturation and activation of NK and T cell responses. IL-21 was found to inhibit the IL-15-dependent expansion of both resting NK cells and those that had undergone prior stimulation. On previously activated NK cells, IL-21 failed to sustain viability but triggered a burst of cytotoxicity and IFNγ production. IL-21 also blocked the IL-15-driven, TCR-independent expansion of CD44^{hi} CD8⁺ cells. In contrast, IL-21 enhanced proliferation, cytotoxic activation, and IFNγ production by antigen-specific T cells. None of these effects were seen in IL-21R^{-/-} mice, confirming the requirement for this receptor chain in mediating cellular responses to IL-21.

IL-21R^{-/-} mice had normal numbers of mature peripheral NK cells, capable of full activation. This result is surprising in view of findings by Parfitt-Novak et al. (2000) that IL-21 potentiates IL-15- and Flt3L-induced NK cell expansion from progenitors in human bone marrow. Indeed, a role in NK cell development would be difficult to reconcile with activated, mature T cells being the only known source of IL-21 (Parfitt-Novak et al., 2000), as the maturation of NK cells in T cell-deficient athymic (Nassiry and Miller, 1987), RAG^{-/-} (Shinkai et al., 1992), and SCID (Dorshkind et al., 1985) mice argues against any critical requirement for a T cell-derived factor in NK cell development. Previous studies have shown that mice rendered deficient in IL-15 (Kennedy et al., 2000) and its receptor components (Di Santo et al., 1995; Suzuki et al., 1997; Lodoce et al., 1998) or Flt3L (McKenna et al., 2000) have profoundly reduced NK cell numbers, underscoring the critical role of these agents in NK cell development. Other cytokines, IL-2 and ckit ligand (SCF), play an auxiliary role. Both can synergize with Flt3L to drive NK cell development from bone marrow progenitors in vitro (Muensch et al., 2000; Kozlak et al., 1996) or when administered in vivo (Fehniger et al., 1997), but mice lacking IL-2 (Kundig et al., 1993) or ckit (W/Wv mice) (Seaman and Tatal, 1981; Colucci and Di

Santo, 2000) have NK cells, albeit at reduced number and activity. Vosshenrich and Di Santo (2001) have speculated that because IL-21R utilizes γc (Asao et al., 2001) and because mice lacking γc have an even more profound reduction in NK cell numbers (Di Santo et al., 1995) than those lacking IL-15Ra (Lodolce et al., 1998), IL-21 could be a key factor in promoting NK cell development *in vivo*. Although we cannot rule out a role in human NK cell development, our finding of normal NK cell numbers and full cytolytic potential in IL-21R^{-/-} mice indicates that IL-21, acting through this receptor chain, is neither essential nor regulatory for NK cell maturation *in mice*.

Nevertheless, IL-21 was able to influence NK cell viability and function, in a manner that discriminated between resting and activated cells. Although RNase protection analysis confirmed expression of IL-21R chain transcripts in both resting and activated NK cells (data not shown), IL-21 enhanced effector function only when used to restimulate NK cells following their initial activation *in vivo* with poly I:C, or *in vitro* with IL-15. In contrast, IL-21 inhibited the IL-15-mediated expansion of NK cells under all conditions tested. In this regard, IL-21 is distinct from the related cytokines IL-2 and IL-15, both of which are able to induce proliferation and cytolytic activation of resting NK cells (Carson et al., 1997; London et al., 1988). Cells from IL-21R^{-/-} mice were fully able to undergo initial activation in response to poly I:C *in vivo* or IL-15 *in vitro* but showed no enhancement of function upon restimulation with IL-21. The ability of IL-21 to enhance effector function only of previously activated NK cells may reflect differential expression of alternative IL-21 receptor chains, signaling molecules, or receptor-induced transcription factors upon initial NK cell activation. Although the basis for the differential IL-21 responsiveness of resting versus activated NK cells remains to be determined, the potential of IL-21 to discriminate between them may be important *in vivo*. A recent report by Yokoyama and colleagues showed that murine NK cells responding early in the course of virus infection are activated nonspecifically, whereas those that persist late into infection require more specific activation signals (Dokun et al., 2001). The selectivity of IL-21 for NK cells that have undergone an initial response could be one mechanism by which those cells that persist late into infection continue to receive activation signals, while the ability of IL-21 to block expansion of resting NK cells could be a mechanism to prevent further recruitment of resting NK cells to the response.

For both resting and activated NK cells, IL-21 alone did not sustain viability and blocked survival but not cytotoxicity induced by IL-15. The observation that growth inhibition was absent in IL-21R^{-/-} mice argues that IL-21R is required to mediate this effect. Because IL-21 blocked NK cell growth in response to IL-2 as well as IL-15 (data not shown) and all three cytokines utilize the γc receptor chain (Asao et al., 2001), one possible explanation is that IL-21 blocked growth effects by competing for a limited pool of γc receptor chains. This type of inhibition should be overcome by addition of higher amounts of IL-15. In our experiments, however, IL-21 blocked NK cell outgrowth at all doses of IL-15 to which the NK cells responded, inconsistent with a model of simple competition between IL-21 and IL-15 for γc chain

interactions. An additional possibility is that the proapoptotic effects of IL-21 prevail over the growth-promoting effects of IL-15, even though the pathways leading to apoptotic versus growth signals are separate, as has been outlined for IL-2 effects on T cells (Van Parijs et al., 1999).

Following an initial activation event *in vivo* or *in vitro*, subsequent challenge with IL-21 greatly enhanced both NK cell cytolytic activity per cell and IFN γ production. This functional activation is necessarily transient, because even as IL-21 promoted NK cell effector responses, it antagonized viability. When exposed to IL-21, activated NK cells underwent apoptosis within 2 days. Given the rapid, potent, and relatively nonspecific nature of their responses, there is a clear biological imperative to have control over expansion of NK cells. This normally occurs during the course of an immune response when abatement of NK cell activation coincides with the emergence of antigen-specific T cells (Biron et al., 1999). While inhibitory receptors can prevent inappropriate activation, few agents have been described that reduce NK cell proliferation once it has been initiated. The T cell-dependent release of TGFB is one such mechanism (Su et al., 1993), but clearly others exist (Pierson et al., 1983). Recently, Loza and Perussia (2001) have demonstrated that human NK cells undergo stepwise maturation from IL-13-producing (NK1) to IFN γ -producing (NK1) effectors, whose activation is followed by terminal differentiation and apoptosis. Generation of IFN γ -producing NK cells was stimulated by the mono-*cyte-derived factor*, IL-12, and slowed by the T cell-derived agent, IL-4 (Loza and Perussia, 2001), suggesting the possibility that emergence of activated T cells feeds back to limit recruitment of NK cells into the immune response. Our findings with IL-21, another T cell-derived cytokine, suggest an additional mechanism whereby activated T cells may influence NK cell maturation. By blocking responsiveness of NK cells to the growth-promoting effects of IL-15 while acting as a potent IFN γ inducer, IL-21 may be a key regulator of NK cell functional status by promoting the terminal steps of NK cell maturation.

Antagonism of IL-15 function was also apparent in IL-21 effects on CD8 $+$ T cells expressing a memory-associated phenotype, CD44 d . As described by Sprent and colleagues, a small percentage of cells which can respond to IL-15 in the absence of TCR signalling arises following contact with antigen in the mouse and persists *in vivo* in the absence of ongoing antigenic stimulation (Zhang et al., 1998). Treatment with IL-15 *in vivo* or *in vitro* selectively induces proliferation of CD44 d CD8 $+$ T cells in an antigen-nonspecific manner (Sprent and Surh, 2001). Although cells with this phenotype initially arise primarily following encounter with antigen (Sprent and Surh, 2001), their subsequent TCR-independent, cytokine-mediated reactivation displays functional characteristics allied with the innate immune response. They undergo bystander proliferation *in vivo* in response to type I interferon or poly I:C (Tough et al., 1998), expand within the first 2 days of virus infection (Turner et al., 2001), proliferate in response to LPS administration (Tough et al., 1997), and produce IFN γ *in vivo* within hours of bacterial infection (Lertmemongkolchai et al., 2001). In our studies, addition of IL-21 inhibited the IL-

IL-15-mediated *in vitro* expansion of CD44^{hi} CD8⁺ T cells from wild-type but not IL-21R^{-/-} spleen. It has yet to be determined whether this decreased expansion is due to prevention of IL-15-induced proliferation or is a pro-apoptotic effect of IL-21 similar to that of IL-2, which may override the growth signals of IL-15 to induce apoptosis in T cells (Li et al., 2001). Including CD44^{hi} CD8⁺ T cells (Ku et al., 2000). Decreased IL-15 responsiveness of CD44^{hi} CD8⁺ T cells mediated by the T cell activation product, IL-21 (Parrish-Novak et al., 2000), may be one mechanism whereby cytokine-driven "bystander" T cell proliferation is reduced once specific immunity emerges. A recent report that CD44^{hi} CD8⁺ T cells undergo apoptosis-mediated attrition upon induction of TCR-mediated anti-viral immunity in LCMV infection (McNally et al., 2001) is consistent with this hypothesis and suggests a role for IL-21.

Whereas IL-21 failed to support expansion of either NK cells or cytokine-activated, TCR-independent CD44^{hi} CD8⁺ T cells, it delivered a potent TCR-dependent accessory signal for T cell responses to alloanigen. Both the allo-specific proliferation of freshly isolated T cells and secondary effector responses, including cytotoxicity and IFN γ production, were stimulated by IL-21. Potentiation of TCR-mediated responses was also apparent in the enhanced proliferation of both thymic and peripheral T cells in response to suboptimal concentrations of anti-CD3 with IL-21. To underscore the essential role of IL-21 in these costimulatory responses, cells from IL-21R^{-/-} mice were used in an allogeneic MLR and did not display IL-21-mediated potentiation of allogeneic T cell responses. While this might indicate that IL-21R^{-/-} cells are somehow impaired in antigen specificity or in interaction with APCs, the finding that these cells had full lytic capacity against allogeneic targets confirms their antigen-recognition potential. These observations implicate IL-21 as a potent inducer of CD8⁺ effector mechanisms in response to allogeneic stimulation and suggest that in the presence of IL-21, the expansion and effector mechanisms of antigen-specific T cells would be greatly enhanced, even as NK and antigen-nonspecific T cell responses were diminished.

In summary, these studies show that IL-21R is not required for the development of NK cells in the mouse, but is necessary to mediate all NK cell and T cell responses to IL-21 that were examined. IL-21 reduced survival of both resting and activated NK cells, even while promoting the effector function of those NK cells that had undergone initial activation *in vivo* or *in vitro*. IL-21 also inhibited the proliferation of TCR-independent CD44^{hi} CD8⁺ T cells in response to IL-15. In contrast, it provided a potent accessory signal for anti-CD3 or antigen-dependent T cell proliferation and effector function. During the course of an immune response, the development and mobilization of antigen-specific T cells coincides with diminished innate responses. This transition, which involves a decrease in NK cell numbers and concomitant T cell expansion (Biron et al., 1999), may be initiated by the presence of activated, mature T cells. As a product of activated T cells that functions to augment antigen-specific T cell responses while antagonizing NK cell survival, IL-21 may be a key facilitator of this transition.

Experimental Procedures

Targeting the IL-21R Gene by Homologous Recombination and Generation of IL-21R^{-/-} Mice

A 400 bp SphI/EcoRV cDNA fragment of IL-21R corresponding to 5' coding regions (aa 2-135) was used as a probe to screen a Stratagene (La Jolla, CA) C57BL/6 mouse liver genomic library for the IL-21R gene. Four clones were isolated from screening 1 × 10⁶ colony forming units. One clone of approximately 13 kb was partially sequenced and found to contain two exons corresponding to the signal sequence region of murine IL-21R. The first leader exon (MPRGPVAA~~LLL~~LHG) was targeted for deletion by replacing it with a neomycin resistance cassette (figure 1A). A 3.8 kb Avr2 fragment 5' to the first leader exon and a 2.0 kb SacI/HindIII intronic fragment 3' to the first leader exon were ligated, 5' and 3', respectively, via linkers (Avr2/20bp1 and BamH1/SacI) to the 1.1 kb XbaI/BamI neomycin cassette and subcloned into pTK(SQ).

IL-21R^{-/-} mice were generated by targeting the IL-21R gene in the J12 embryonic stem cell line (C57BL/6 origin), injecting clones into blastocysts, and transferring to pseudopregnant B6CBA F1 females. Resulting male chimeras were bred to BALB/c females, and offspring were analyzed by PCR and Southern blotting for germline transmission of the mutant allele. Mice heterozygous for the IL-21R mutation (IL-21R^{+/−}) were intercrossed to yield homozygous offspring (IL-21R^{−/-}) on the BALB/c × C57BL/6 background and subsequently bred onto the C57BL/6 background. The lack of IL-21 expression in IL-21R^{−/-} mice was confirmed by PCR analysis using total RNA isolated from tail bleeds and analysis of amplified products by Southern blot. Mice on the BALB/c × C57BL/6 background were used for initial characterization and those on the C57BL/6 background were used for functional studies, unless otherwise noted. Data are presented for mice aged between 8–12 weeks.

Murine IL-21

The 441 bp coding region of mIL-21 cDNA encoding a protein of 148 amino acids (Parrish-Novak et al., 2000) was inserted into the COS-1 expression vector, pED4c. COS transfectants were grown in DMEM containing 10% FBS in 10% CO₂. Cells were plated in serum-free DMEM for 30 hr post-transfection and IL-21-containing supernatant was collected 24 and 48 hr later. The supernatant was concentrated 50× by Amicon filtration. PMSF and EDTA were added to prevent proteolysis. One unit of activity was defined as the concentration of supernatant required to induce 60% maximal proliferation of Ba/F3 cells transfected with IL-21R. When tested in the absence of exogenous IL-3, proliferation of these transfectants is IL-21 dependent. Mock-transfected COS supernatant prepared and concentrated in parallel with IL-21 was used as a control.

NK Cell Activation *In Vitro*

Spleen cell suspensions were depleted of RBC with ammonium chloride and plated in RPMI containing 10% FBS, 50 U/ml penicillin, 50 µg/ml streptomycin, 2 mM L-Glutamine, and 50 µg/ml β-mercaptoethanol with 10–50 ng/ml recombinant human IL-15 (R&D Systems, Minneapolis, MN), 12.5 U/ml recombinant mouse IL-21, or an equal volume of COS mock control supernatant. This dose of IL-21 had been shown to have maximal activity for the inhibition of NK cell outgrowth (data not shown). Cells were cultured at 5% CO₂ for 7 days, with a second dose of IL-15 and IL-21 or COS mock control added on day 4. On day 7, supernatant and adherent cells were assayed for cytotoxicity, IFN γ production, or cell surface phenotype.

NK Cell Activation *In Vivo*

Mice were injected i.p. with 0.15 ml PBS containing 1 mg/ml poly-D,L-histidine-polyacrylic acid (poly IC; Sigma) or PBS control. Spleens were harvested 1.5 days later, and cells were used in a 51-Cr release assay against YAC-1 targets.

NK Cytotoxicity

YAC-1 target cells (American Type Culture Collection, Manassas, VA) were incubated for 1 hr with sodium 51-chloroate (20 µCi/1 × 10⁶ cells; New England Nuclear, Boston, MA), washed, and plated with effector cells at the indicated effector:target cell (E:T) ratio.

After 5 hr incubation at 37°C, supernatants were harvested, and radioactivity was determined in a γ counter. Maximum release was determined by lysing YAC-1 target cells with Triton X-100. Spontaneous release was determined as 51-Cr released into the supernatant of YAC-1 targets incubated in the absence of effectors. Percent specific lysis was calculated according to the formula: (test - spontaneous release)/(total - spontaneous release) \times 100.

IFN γ Production

Spleen cells treated with IL-15 or IL-21 as described above were washed and repleted at 5×10^6 /ml for 24 hr with the indicated concentration of recombinant murine IL-12 (Genetics Institute, Cambridge, MA). IFN γ levels were assayed by Quantikine mouse IFN γ ELISA kit (R&D Systems; detection limit = 10 pg/ml).

Thymocyte Proliferation

Single-cell thymocyte suspensions were cultured in DME containing 10% FBS, 50 U/ml penicillin, 50 μ g/ml streptomycin, 2 mM L-glutamine, and 50 μ M β -mercaptoethanol, in the presence or absence of 25 U/ml IL-21. Cells were primed at 2×10^6 /well in 96-well flat-bottom plates coated with 1 μ g/ml anti-CD3 (mAb 2C11; Pharmingen). On day 3, cultures were pulsed with 0.5 μ Ci/well [3 H]thymidine (Amersham Biosciences, Piscataway, NJ) and harvested 5 hr later onto glass fiber filter mats. [3 H]thymidine incorporation was determined by liquid scintillation counting.

Mixed Lymphocyte Reaction

T cells were purified from lymph nodes of wild-type or IL-21R $^{-/-}$ mice using negative selection columns (R&D Systems). For primary proliferation assays, T cells (2×10^6 /ml) were cultured in 96-well plates coated with 1 μ g/ml anti-CD3 (mAb 2C11, Pharmingen) or with erythrocyte-depleted, irradiated B10.Br splenocytes (3×10^6 /ml) and the indicated cytokines for 3 days, then pulsed for 12 hr with 1 μ Ci [3 H]thymidine. For effector function assays, purified LN T cells (5×10^6 /ml) were primed with erythrocyte-depleted, irradiated B10.Br splenocytes (2×10^6 /ml) in the presence of the indicated cytokines: rIL-2 (20 U/ml; R&D Systems), rmIL-21 (10 U/ml), or an equivalent volume of COS mock control supernatant. After 6–7 days, cells were harvested, washed, and used in a 4 hr CTL assay with B10.Br or syngeneic spleen blasts as target cells. The splenic blasts prepared by 48 hr treatment of erythrocyte-depleted splenocytes with 10 μ g/ml LPS and DXS (Sigma). Percent specificity lysis was calculated as for NK cytotoxicity. For IFN γ production, T cells "primed" with alloantigen and the indicated cytokines were washed, counted, and restimulated (2.5 $\times 10^6$ /ml) with irradiated B10.Br splenocytes (1×10^6 /ml) for 40 hr. Supernatants were assayed for IFN γ levels by ELISA (R&D Systems).

Flow Cytometry and Quantification of Lymphocyte Subsets

Cells were resuspended in PBS containing 1% BSA and incubated 15 min at 4°C with Fc block (Pharmingen), followed by biotinylated antibody to various cell surface markers or appropriate isotype control (Pharmingen). Cells were washed in the same buffer, then incubated 15 min at 4°C with the appropriate FITC-labeled or PE-labeled antibody to cell surface markers or isotype control (Pharmingen) and streptavidin-Red 670 (Gibco Life Technologies). Fluorescein TUNEL staining was performed using the In Situ Cell Death Detection Kit (Roche Diagnostics, Mannheim, Germany). Analysis was performed on a FACScan with CellQuest software (Becton-Dickinson, San Jose, CA). In all cases, viable cells were gated based on forward and side scatter. For quantitation of various cell subsets, the percentage of cells in that subset was determined by flow cytometry and multiplied by the total number of lymphocytes per culture.

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Pharmacokinetics of recombinant human interleukin-2 in advanced renal cell carcinoma patients following subcutaneous application

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Aims The aim of the study was to investigate the pharmacokinetics of recombinant human interleukin-2 (rhIL-2) in patients with metastatic renal cell carcinoma following different subcutaneous (s.c.) administration regimens.

Methods RhIL-2 was administered subcutaneously to 10 patients according to two different dosing regimens: group A received 20×10^6 IU m⁻² once daily and group B 10×10^6 IU m⁻² twice daily (every 12 h). Additionally, in all patients the influence of soluble interleukin-2 receptor (sIL-2R) on the pharmacokinetics of rhIL-2 was investigated.

Results The mean area under the serum concentration-time curve to 24 h (AUC(0,24 h)) was $627 \text{ IU ml}^{-1} \text{ h}$ in treatment group A and $1130 \text{ IU ml}^{-1} \text{ h}$ ($P=0.029$) in treatment group B. In both study groups C_{\max} and AUC(0,12 h) were not significantly different. Seventy-two hours after the beginning of s.c. rhIL-2 therapy the sIL-2R increased significantly ($P=0.016$), and sIL-2R levels over 1200 pmol l^{-1} seemed to reduce the AUC.

Conclusions In patients with metastatic renal cell cancer administration of 20×10^6 IU m⁻² of rhIL-2 s.c. in two daily doses (10×10^6 IU m⁻² every 12 h) provides better bioavailability and is preferable to the single dose administration.

Keywords: interleukin-2, pharmacokinetics, soluble interleukin-2 receptor, subcutaneous

Introduction

The use of recombinant human interleukin-2 (rhIL-2) has been recommended as the best current therapy for advanced renal cell carcinoma [1, 2]. RhIL-2 was found to exert its antitumour activity via indirect effects on the immune system, including the activation and expansion of cytotoxic T-lymphocytes and natural killer cells, and the secretion of secondary cytokines such as interferon-γ and TNF-α [3, 4]. The immune modulatory capacity has been described for the i.v. administration of high dose rhIL-2, which was associated with severe adverse effects, including capillary leak-related weight gain, hypotension, malaise, fever, and chills. Subcutaneous (s.c.) rhIL-2 at doses far below the maximum tolerable dose is therapeutically effective while treatment-related toxicity is reduced [5–7]. The pharmacokinetics of intravenously and intramuscularly administered rhIL-2 are well known [8–10], but only limited data on the pharmacokinetics after subcutaneous administration are available and information on the comparison of various dose regimens of s.c. rhIL-2 is lacking.

During systemic administration of rhIL-2 in humans, elevated soluble IL-2 receptor levels have been found [11]. The soluble IL-2 receptor (sIL-2R) differs from its membrane-bound counterpart with respect to size, binding capacity and ligand specificity. The soluble form of the

human IL-2 receptor is a glycosylated protein with a molecular weight between 35 and 50 kDa [12]. It binds to IL-2 with low affinity ($K_d: 10 \text{ nmol l}^{-1}$) which is comparable with the affinity to the membrane bound α-chain of the IL-2-receptor [13]. The α-chain is expressed on activated T cells which combines with the β-and γ-chains to constitute a high-affinity IL-2R with a 1000-fold higher affinity to IL-2 than presented by the sIL-2R [14–17]. It has been hypothesized that the competition between the membrane-bound and the soluble receptor for IL-2 causes inhibition of IL-2 dependent mechanisms. However, so far, no data are available on the influence of sIL-2R on IL-2 pharmacokinetics.

The present study attempts to provide a more detailed analysis of the pharmacokinetics of subcutaneous rhIL-2 in patients with metastatic renal cell carcinoma comparing different dosing regimens. In addition, pharmacokinetic data of s.c. rhIL-2 were analysed in relation to different concentrations of the sIL-2 receptors.

Methods

Patients and treatment

The study cohort consisted of 10 patients (mean age 56 ± 9 years) treated at our institution with histologically confirmed metastatic renal cell cancer in a clinically progressive stage; all patients had a Karnofsky performance status $> 70\%$. No chemotherapy or immune modulatory therapy was per-

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formed for at least 4 weeks prior to this protocol. After obtaining written informed consent patients were treated with s.c. rhIL-2 (Chiron, Emeryville, USA). Two different priming doses of rhIL-2 ($1 \text{ IU} = 2.916 \text{ pg}$) were administered in the first week of therapy on 3 consecutive days: seven patients (mean age 58 ± 8 years; mean body weight $71 \pm 11 \text{ kg}$) received $20 \times 10^6 \text{ IU m}^{-2}$ rhIL-2 s.c. once daily (group A) and three patients (mean age 50 ± 10 years; mean body weight $69 \pm 12 \text{ kg}$) received $10 \times 10^6 \text{ IU m}^{-2}$ rhIL-2 s.c. twice daily at a 12 h interval (group B). This study was approved by the institutional ethical committee of the Medizinische Hochschule Hannover.

Sampling

Patients' sera were obtained and stored at -20°C until analysis. The samples for rhIL-2 determination were collected immediately before (0 time) and 0.5, 1, 1.5, 2, 4, 6, 8, 10, 12, 14, 16, 20, 22 and 24 h after rhIL-2 s.c. injection. Additional sera were prepared for the determination of the soluble interleukin-2 receptor concentration prior to rhIL-2 injection and at least every 4 h during 24 h in all 10 patients. Concentrations of rhIL-2 and sIL-2R were determined in two patients of each treatment group during 3 days of treatment and in one patient of each treatment group during 2 days of treatment.

Interleukin-2 assay

To assay the serum concentration of rhIL-2 a commercial standard cytokine ELISA kit (Medgenix, Ratingen, Germany) was used and performed according to the manufacturer's guidelines. Standard samples, which were contained in the ELISA kit, were used. The lower limit of quantification was 0.5 IU ml^{-1} . A three-run validation was performed to verify the precision and accuracy of the assay. The precision was expressed as the coefficient of variation (CV) of the measured concentrations. The within-run precision ($n=5$) for the assay at nominal concentrations of 10 , 35 and 70 IU ml^{-1} was 3.6% , 5.7% , and 3.9% , respectively. The between-run precision of the assay at these concentrations was 6.1% , 7.5% , and 7.7% , respectively.

ELISA for soluble IL-2R (sIL-2R)

Soluble IL-2R levels were determined using a standard two-step sandwich assay (Immunotech, Marseille, France), as described previously in detail [18]. The amount of sIL-2R per sample was calculated by plotting the absorbance values against a sIL-2R standard curve. Normal donor values ranged from 25 to 115 pmol l^{-1} ($1 \text{ pmol l}^{-1} = 42 \text{ pg ml}^{-1}$). The lower limit of quantification was 5 pmol l^{-1} . The within-run precision was assessed by performing an analysis at three defined concentrations (25 , 400 , and 800 pmol l^{-1} ; $n=5$). The coefficient of variation was 6.6% , 4.9% , and 5.3% , respectively. The between-run precision of the assay at the above mentioned concentrations was 11.4% , 7.9% , and 9.5% , respectively.

Pharmacokinetic analysis

Compartment independent pharmacokinetic parameters of rhIL-2 were evaluated using the TopFit version 2.0 software [19]. The maximal concentration (C_{\max}), the corresponding time (t_{\max}) and area under the curve ($\text{AUC}(0,24 \text{ h})$) were determined. The elimination half-time ($t_{1/2,\alpha}$) was calculated from the log-linear terminal slope ($4-24 \text{ h}$ in patients who received $20 \times 10^6 \text{ IU m}^{-2}$; and $4-12 \text{ h}$ in patients who received $10 \times 10^6 \text{ IU m}^{-2}$ twice daily). For statistical analysis the Wilcoxon's rank sum test was used to compare both treatment regimens. Data are given as means \pm s.d.

Results

Subcutaneous administration of $20 \times 10^6 \text{ IU m}^{-2}$ rhIL-2 once daily

Seven patients of study group A were treated with s.c. administration of $20 \times 10^6 \text{ IU m}^{-2}$ once daily. The pharmacokinetic profile of the mean serum rhIL-2 concentration is presented in Figure 1(a). The pharmacokinetic parameters including t_{\max} , C_{\max} , $t_{1/2,\alpha}$ and AUC are summarized in Table 1. In group A the mean $\text{AUC}(0,24 \text{ h})$ -value was $627 + 153 \text{ IU ml}^{-1} \text{ h}$ and the apparent harmonic mean $t_{1/2,\alpha}$ -value $5.1 + 1.1 \text{ h}$. Mean C_{\max} was $72 + 20 \text{ IU ml}^{-1}$ which was reached at a time of $4.0 + 1.2 \text{ h}$. The mean AUC value ($\text{AUC}(0,12 \text{ h})$) was $501 + 125 \text{ IU h ml}^{-1}$ (Table 1).

Subcutaneous administration of $10 \times 10^6 \text{ IU m}^{-2}$ rhIL-2 every 12 h (twice daily)

Patients of the treatment group B received $10 \times 10^6 \text{ IU m}^{-2}$ of rhIL-2 s.c. every 12 h (three patients). In group B C_{\max} of rhIL-2 in the first 12 h period amounted to $89 \pm 25 \text{ IU ml}^{-1}$ at a t_{\max} of $4.0 \pm 0 \text{ h}$. During the second 12-h period after the administration of $10 \times 10^6 \text{ IU m}^{-2}$ rhIL-2 the mean C_{\max} was $82 \pm 31 \text{ IU ml}^{-1}$ at a t_{\max} of $2.7 \pm 1.7 \text{ h}$ (shown in Table 2). The mean $\text{AUC}(0,12 \text{ h})$ of the first 12 h was $576 \pm 126 \text{ IU ml}^{-1} \text{ h}$, that of the second 12 h period ($\text{AUC}(12,24 \text{ h})$) was $554 \pm 184 \text{ IU ml}^{-1} \text{ h}$. Both mean AUC values ($\text{AUC}(0,24 \text{ h})$) added up to $1130 \text{ IU ml}^{-1} \text{ h}$. The pharmacokinetic profile of the mean rhIL-2 concentrations is shown in Figure 1b.

Comparison of the pharmacokinetic data of both study groups

We investigated the influence of the dosage of rhIL-2 on the C_{\max} , t_{\max} and AUC values after administration of single dose of $10 \times 10^6 \text{ IU m}^{-2}$ or $20 \times 10^6 \text{ IU m}^{-2}$ rhIL-2 s.c. 4.0 h after rhIL-2 administration t_{\max} was reached in both study groups. The mean C_{\max} values of group A and group B (72 IU ml^{-1} vs 89 IU ml^{-1}) were not significantly ($P=0.209$) different. We also compared the mean $\text{AUC}(0,12 \text{ h})$ values of both study groups ($501 \text{ IU ml}^{-1} \text{ h}$ vs $576 \text{ IU ml}^{-1} \text{ h}$) and found no significant difference ($P=0.305$). On the contrary, the $\text{AUC}(0,24 \text{ h})$ following a twice daily administration of $10 \times 10^6 \text{ IU m}^{-2}$ rhIL-2 was significantly ($P=0.029$) higher (nearly twice as high) than the $\text{AUC}(0,24 \text{ h})$ after administration of ($20 \times 10^6 \text{ IU m}^{-2}$) in one daily cumulative dose.

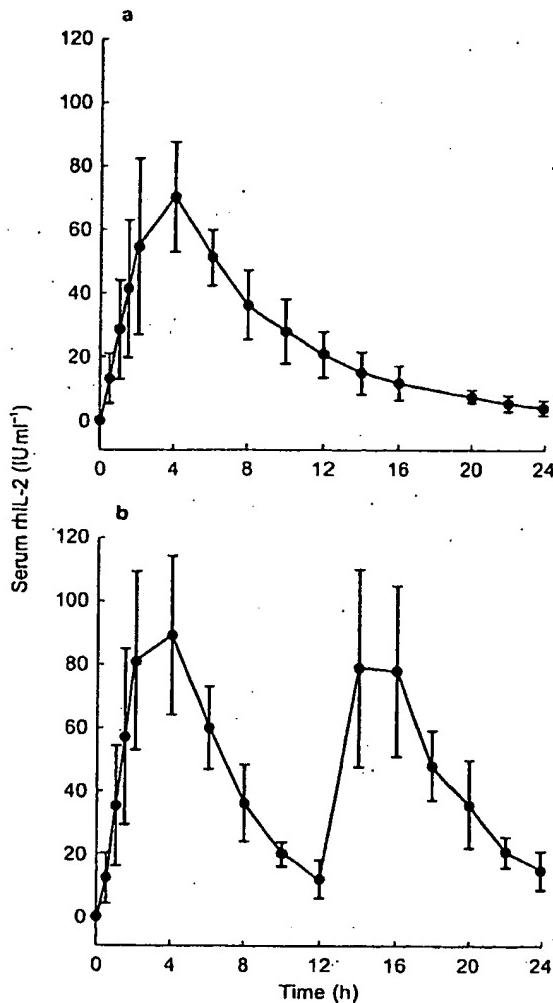


Figure 1 Pharmacokinetics of rhIL-2 in patients with metastatic renal cell cancer treated subcutaneously. Serum levels of rhIL-2 were measured with ELISA and are expressed as mean \pm s.d. (a) Mean levels in seven patients receiving 20×10^6 IU m^{-2} s.c. as single injection. (b) Mean levels in three patients receiving 10×10^6 IU m^{-2} s.c. twice every 12 h.

Soluble IL-2 receptor (sIL-2R)

The serum concentrations of the sIL-2R of all 10 patients are shown in Figure 2. Prior to immunotherapy three patients exhibited normal sIL-2R levels ($25-115$ pmol l^{-1}), while seven patients had elevated levels, which ranged from 182 to 828 pmol l^{-1} . There was no significant difference between both study groups prior to the treatment ($P=0.425$), 24 h after the first injection of s.c. rhIL-2 ($P=0.425$) and 48 h after start of therapy ($P=0.275$). During rhIL-2 therapy the concentrations of the sIL-2R increased during the first 48 h continuously, but not significantly, in treatment group A from 286 pmol l^{-1} up to 614 pmol l^{-1} ($P=0.237$) and in treatment group B from 325 pmol l^{-1} up to 955 pmol l^{-1} ($P=0.109$). Three days after s.c. administration of rhIL-2 the sIL-2R seemed to increase distinctly (group A: from 286 pmol l^{-1} up to 1168 pmol l^{-1} and group B: from 325 pmol l^{-1} up to 1934 pmol l^{-1}): The sIL-2R was measured only in two patients of each treatment group up to 3 days after the beginning of rhIL-2 therapy, therefore we could not calculate significance using the Wilcoxon test. When summarizing both study groups we could show that the sIL-2R increased significantly ($P=0.016$) 72 h after the beginning of rhIL-2 treatment.

We also evaluated the influence of the increase of sIL-2R concentrations on the values of AUC(0,24 h). During the first 48 h after rhIL-2 administration, the increase of the sIL-2R had no apparent influence on the AUC values. After more than 48 h, there was a trend towards a decrease in AUC in those patients with sIL-2R concentrations over 1200 pmol l^{-1} ($n=3$), although this failed to reach statistical significance. The serum levels of sIL-2R and rhIL-2 of one typical patient who received 10×10^6 IU m^{-2} rhIL-2 s.c. are shown in Figure 3.

Discussion

In this study we have determined the pharmacokinetics of two different dose regimens of subcutaneous rhIL-2 administration in patients with metastatic renal cell cancer. Since ELISA assays have been employed, the current results do not necessarily correspond to the availability of biologically effective IL-2. We have shown for the first time that administration of 10×10^6 IU m^{-2} rhIL-2 s.c. twice daily (every 12 h) results in a significantly higher ($P=0.029$) total AUC(0,24 h) than s.c. administration of 20×10^6 IU m^{-2} once daily. Although pharmacokinetic parameters are best

Table 1 Pharmacokinetic parameters of a single injection of rhIL-2 at 20×10^6 IU m^{-2} s.c. in seven patients with metastatic renal cell cancer.

Number	t_{max} (h)	C_{max} (IU ml^{-1})	$t_{1/2,\alpha}(4,24\text{ h})$ (h)	$AUC(0,12\text{ h})$ (IU $ml^{-1}\text{ h}$)	$AUC(0,24\text{ h})$ (IU $ml^{-1}\text{ h}$)
1	2.0	105	3.1	647	748
2	4.0	80	4.8	649	829
3	6.0	45	6.5	375	501
4	4.0	52	5.3	366	450
5	4.0	66	6.2	483	657
6	4.0	75	5.0	583	732
7	4.0	80	4.9	401	450
Mean \pm s.d.	4.0 ± 1.2	72 ± 20	5.1 ± 1.1	501 ± 125	627 ± 153

Number	Time (h)	t_{max} (h)	C_{max} (IU ml $^{-1}$)	$t_{1/2}(4, 12 \text{ h})$ (h)	AUC (IU ml h $^{-1}$)
8	0–12	4.0	67	2.9	446
9		4.0	116	2.1	698
10		4.0	83	3.5	585
Mean \pm s.d.		4.0 \pm 0	89 \pm 25	2.8 \pm 0.7	576 \pm 126
8	12–24	2.0	48	3.5	369
9		4.0	88	3.1	555
10		2.0	110	3.6	737
Mean \pm s.d.		2.7 \pm 1.7	82 \pm 31	3.4 \pm 0.3	554 \pm 184
	0–24				576 \pm 554 = 1130

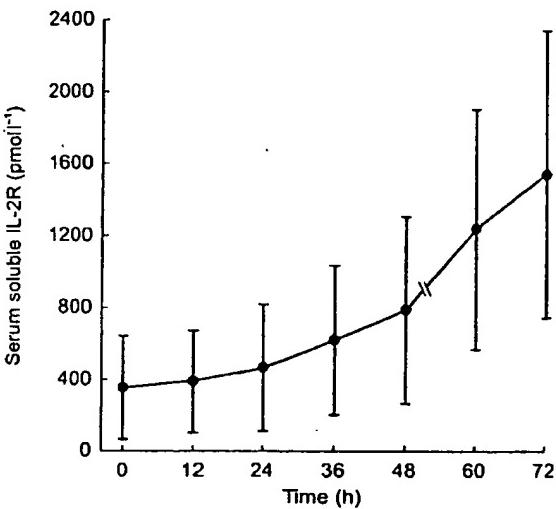


Figure 2 Serum concentrations (mean \pm s.d.) of soluble IL-2 receptor measured by ELISA during rhIL-2 therapy ($n=6$ until 48 h, $n=4$ until 72 h).

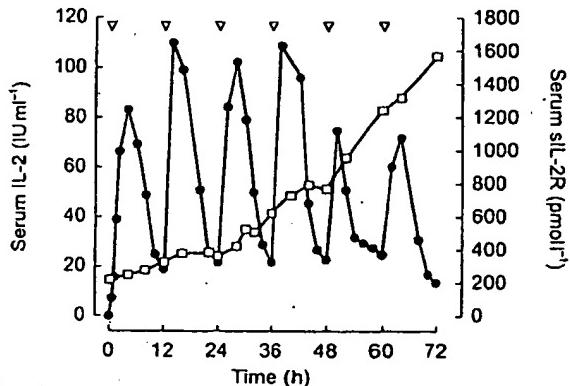


Figure 3 Serum levels of rhIL-2 (●) and soluble IL-2 receptor (□) after subcutaneous application ($10 \times 10^6 \text{ IU m}^{-2}$ s.c. every 12 h for 3 days, ▼) in one typical patient.

determined from i.v. bolus and infusion data there are several reports of IL-2 pharmacokinetics following i.m., i.p. and s.c. administration [8–10]. Until now no previous study described the pharmacokinetic data of different dose regimens of subcutaneous rhIL-2 administration. Only

Table 2 Pharmacokinetic parameters of two consecutive injections of rhIL-2 at $10 \times 10^6 \text{ IU m}^{-2}$ s.c. every 12 h in patients with metastatic renal cell cancer.

Ettinghausen et al. [20] demonstrated that intraperitoneal (i.p.) injection of rhIL-2 three times a day was more effective than the cumulative IL-2 dose administered daily i.p. or i.v.

Furthermore, we could show, that there was no significant difference between the C_{max} ($P=0.208$) and AUC(0,12 h) ($P=0.305$) values after administration of a single dose of 10×10^6 or $20 \times 10^6 \text{ IU m}^{-2}$ rhIL-2 s.c. Gustavson et al. [10] reported that serum concentrations of rhIL-2 following i.v. administration ($0.1\text{--}30 \times 10^6 \text{ U}$) increased in an apparently dose-proportional manner. However, when administered s.c. ($0.1\text{--}3.0 \times 10^6 \text{ U}$), the increase in serum concentration was less than expected, which may have been due to a dose-dependent reduction in bioavailability for s.c. administered rhIL-2 [10]. We suggest that this effect is caused by an incomplete release of rhIL-2 from the subcutaneous injection site, hence, administration of doses higher than $10 \times 10^6 \text{ IU m}^{-2}$ rhIL-2 did not lead to significantly increased bioavailability. This observation is consistent with the results of other studies [21, 22] whereby recombinant human granulocyte-macrophage colony-stimulating factor (rhGM-CSF) was administered s.c. in different doses. Stute et al. [21] described that C_{max} and the AUC did not increase proportionally to the dose of s.c. rhGM-CSF; to explain this phenomenon a reduced absorption from the injection site was also hypothesized [21].

Our pharmacokinetic parameters $t_{1/2}=2.8\text{--}5.1 \text{ h}$ and $t_{max}=4.0 \text{ h}$ are compatible with previous pharmacokinetic studies [9, 10]. Konrad et al. [9] used Cetus units (1 Cetus unit = 6 IU); they reported after i.v. bolus administration of a median dose of $20 \times 10^6 \text{ IU m}^{-2}$ rhIL-2 a nearly two-fold higher AUC ($2465 \text{ IU ml}^{-1} \text{ h}$) when compared with the present AUC after s.c. rhIL-2 administration of $20 \times 10^6 \text{ IU m}^{-2}$ in two daily doses ($10 \times 10^6 \text{ IU m}^{-2}$ every 12 h) in our study (AUC(0,24 h) = $1130 \text{ IU h ml}^{-1}$). Comparing s.c. with i.v. bolus administration, the peak levels after s.c. administration were more than 10–100 times lower than immediately after i.v. bolus rhIL-2, but were approximately constant for several hours before gradually decreasing. Therefore, the reported toxicity of i.v. rhIL-2 was much higher in comparison to the s.c. application route [9].

We found elevated sIL-2R levels in seven of 10 patients with renal cell carcinoma prior to rhIL-2 therapy. Elevated sIL-2R levels have been described earlier in patients with advanced renal cell carcinoma [23]. Furthermore, sIL-2R levels are increased in several diseases, mostly in those of

infections [24, 25] or neoplastic character, like malignant melanoma, multiple myeloma, chronic myelogenous leukaemia [23, 26, 27].

After 3 days of s.c. rhIL-2 therapy the sIL-2R concentrations increased significantly ($P=0.016$). During the first 48 h after the start of rhIL-2 treatment the sIL-2R seemed to have no influence on the AUC values, because of the low sIL-2R concentrations. But in parallel to the increase of the sIL-2R concentrations over 1200 pmol l^{-1} a tendency toward reduced AUC amounts could be observed. A potential immune modulatory role of the sIL-2R has been discussed earlier [28], but not without controversy [29, 30]. The main objection against such a role was the low affinity ($K_d: 10 \text{ nmol l}^{-1}$) of the sIL-2R for IL-2, which is 1000-fold lower than the binding affinity of the membrane-bound heterotrimeric receptor complex ($K_d: 0.01 \text{ nmol l}^{-1}$) [16–18, 30–32]. Nevertheless, inhibition of IL-2 driven effects like proliferation of IL-2 dependent mouse CTLL cell line or inhibition of induction of cell-mediated cytotoxicity has been demonstrated by several investigators [30, 33–35].

In addition to the *in vitro* studies, in several clinical studies the putative physiological significance of sIL-2R has been investigated. During systemic administration of rIL-2 in humans, elevated sIL-2R levels have been observed earlier [15]. We described previously that during long-term s.c. rIL-2 treatment both soluble and cell surface IL-2R (CD25) exhibit a significant increase [15]. We could observe a positive correlation between the serum levels of sIL-2R and CD25 cell surface expression on peripheral blood lymphocytes. The quantitative correlation stated for soluble and membrane-bound IL-2R expression may be explained by its subsequent shedding without the transmembrane domain of the CD25 molecule [32].

The exact mechanism of the immune modulatory capacity of sIL-2R *in vitro* and *in vivo* remains still to be clarified. Here, we suggest two different possible mechanisms of immune modulation by the soluble IL-2R depending on its concentration. *In vitro* we could previously demonstrate that the neutralizing capacity for IL-2 driven immune responses (like CTLL-proliferation, cytotoxicity to malignant cell lines) is dose-dependent for IL-2R concentrations up to 100 pmol l^{-1} and is not due to a direct functional interaction between sIL-2R and free IL-2; at this lower concentration, an interaction of sIL-2R with the membrane-bound IL-2R seemed to be possible [18].

Our present *in vivo* data showed a trend towards decreasing AUC(0,24 h) values for sIL-2R concentrations higher than 1200 pmol l^{-1} . Because of the low affinity of sIL-2R in comparison to the membrane-bound IL-2R, a direct interaction between the sIL-2R and free nonbound IL-2 seemed to be possible only at higher concentrations of the sIL-2R.

In conclusion, the pharmacokinetics of s.c. rhIL-2 in patients with metastatic renal cell carcinoma can be summarized as follows:

[A] Split s.c. injection (every 12 h) of rhIL-2 results in a significantly ($P=0.029$) higher total AUC(0,24h) than corresponding single dose administered once daily. [B] There were no significant differences between the C_{\max} and AUC(0,12h) levels after single s.c. administration of 10×10^6

and $20 \times 10^6 \text{ IU m}^{-2}$ of rhIL-2, respectively. [C] AUC(0,24h) following s.c. administration of rhIL-2 in two daily doses was comparable with AUC(0,24h) reported after i.v. bolus rhIL-2. [D] The sIL-2R increased significantly ($P=0.016$) during treatment with rhIL-2, and soluble IL-2R levels over 1200 pmol l^{-1} seemed to cause a decrease of AUC(0,24h).

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IL-21 Activates Both Innate and Adaptive Immunity to Generate Potent Antitumor Responses that Require Perforin but Are Independent of IFN- γ ¹

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IL-21 is a key factor in the transition between innate and adaptive immune responses. We have used the cytokine gene therapy approach to study the antitumor responses mediated by IL-21 in the B16F1 melanoma and MethA fibrosarcoma tumor models in mice. Retrovirally transduced tumor cells secreting biologically functional IL-21 have growth patterns in vitro similar to that of control green fluorescent protein-transduced cells, but are completely rejected in vivo. We show that IL-21 activates NK and CD8⁺ T cells in vivo, thus mediating complete rejection of poorly immunogenic tumors. Rejection of IL-21-secreting tumors requires the presence of cognate IL-21R and does not depend on CD4⁺ T cell help. Interestingly, perforin, but not IFN- γ or other major Th1 and Th2 cytokines (IL-12, IL-4, or IL-10), is required for the IL-21-mediated antitumor response. Moreover, IL-21 results in 50% protection and 70% cure of nonimmunogenic tumors when given before and after tumor challenge, respectively, in C57BL/6 mice. We conclude that IL-21 immunotherapy warrants clinical evaluation as a potential treatment for cancer. *The Journal of Immunology*, 2003, 171: 608–615.

Interleukin-21 and its receptor (IL-21R) have been recently characterized and described (1). Secreted by activated T cells, IL-21 is a 131-aa residue, four-helix bundle cytokine with sequence homologies to IL-2 and IL-15 (1). IL-21R is expressed in lymphoid tissues, in particular by NK, B, T, and dendritic cells, macrophages, and endothelial cells (1) (data not shown). IL-21R has the highest sequence homology to IL-2R β chain and IL-4R α chain (2), which associates with the common γ cytokine receptor chain (γ c)³ (2, 3) upon ligand binding. The widespread lymphoid distribution of IL-21R suggests that IL-21 may potentially play a substantial role in immune regulation. Indeed, in vitro studies have shown that IL-21 significantly modulates the function of B, T, and NK cells (1, 4–6). IL-21 potentiates maturation of NK cell from bone marrow progenitors and activation of peripheral NK cells in human assay systems (1). In murine systems, Kasaian et al. (4) have demonstrated that IL-21 limits ongoing NK cell expansion while promoting NK effector functions and Ag-specific CD8⁺ T cell responses. More recently, Wurster et al. (7) have shown that IL-21 specifically inhibits IFN- γ production from developing Th1 cells and is preferentially expressed by Th2 cells. These data suggest that IL-21 may play a unique role in fine tuning the response of B, T, and NK cells, depending on the type of stimulus and the phenotype of immune cells.

The cytokine gene therapy approach as a form of molecular pharmacology applied to tumor models has contributed significantly to identifying immune responses mediated by cytokines that were previously either unknown or not fully appreciated (8). In this study we have used the cytokine gene-transfer technology in two tumor models and studied the in vivo biology of IL-21. Our results show that IL-21/IL21R interactions have a unique role in sequentially activating both innate and adaptive immune responses against poorly immunogenic tumors, leading to tumor rejection that is perforin (psp) dependent but IFN- γ independent. More important, we demonstrate that IL-21 stimulates potent prophylactic and therapeutic immunity that leads to the cure of tumors.

Materials and Methods

Mice

Female C57BL/6, BALB/c, IL-4^{-/-}, IL-10^{-/-}, IFN- γ ^{-/-}, IL-12^{-/-} (i.e., p35^{-/-}), and psp^{-/-} deficient mice in C57BL/6 background were purchased from The Jackson Laboratory (Bar Harbor, ME). C57BL/6 SCID and nude mice were purchased from Taconic Farms (Germantown, NY). IL-21R^{-/-} mice (4) were maintained at Charles River Breeding Laboratories (Andover, MA). All mice were maintained and treated in accordance with National Institutes of Health and American Association of Laboratory Animal Care regulations.

Tumor cell lines and reagents

B16F1 melanoma cells were maintained in culture in DMEM medium supplemented with 10% heat inactivated FBS, 2% glutamine, and 1% penicillin-streptomycin. MethA fibrosarcoma cells were maintained by i.p. passage in BALB/c mice. B16F1 melanoma-specific tyrosinase related protein-2 (TRP-2) (SVYDFFVWL) (9, 10) and control peptide OVA_{257–264} (SIINFEKL) were synthesized at Wyeih Research (Cambridge, MA). mAbs anti-CD3, anti-CD28, and rat IgG2a and IL-2 cytokine used in this paper were all purchased from BD PharMingen (San Diego, CA).

Generation of IL-21 and green fluorescent protein (GFP)-expressing tumor cells

B16F1 and MethA tumor cells were engineered to express IL-21 and GFP, or only GFP. Retroviral vectors encoding mIL21-IRES-GFP or IRES-GFP were constructed using GFP-RV vector (kindly provided by Dr. K. Murphy, Washington University, St. Louis, MO) (11). High titer retrovirus was

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³ Abbreviations used in this paper: γ c, common γ cytokine receptor chain; GFP, green fluorescent protein; psp, perforin; TRP-2, tyrosinase related protein-2.

obtained by transfecting the 293-vesicular stomatitis virus G ecotropic packaging cell line (12). Spin infections were performed at 1800 rpm for 40 min at room temperature. Cells were infected three times. Tumor cells expressing GFP were enriched by flow sorting, and the purity of GFP-expressing cells was >90%.

In vivo tumor studies

C57BL/6 mice were shaved on the back and injected i.d. with 10^5 B16F1-IL-21 or control B16F1-GFP. BALB/c mice were injected with either 10^6 or 2×10^6 MethA-IL-21 or control MethA-GFP cells. Tumor growth was monitored by measuring perpendicular diameters with a caliper. Mice were sacrificed when the tumors displayed severe ulceration or reached a size of 200 mm^2 . In general, 10 mice per group were used in each experiment, and tumor size averages from each group are shown. Results of this study (except in vivo depletion studies) represent experiments repeated at least two times with similar results. The difference in tumor size between the control and experimental groups was statistically analyzed using Student's *t* test.

In vivo depletion studies

CD4⁺ or CD8⁺ T cell depletion was accomplished by i.p. injecting 400 μg of either anti-CD4 (GK1.5), anti-CD8 (53-6.7) mAbs, or rat IgG isotype control per mouse for three consecutive days before tumor cell injection. Ab injections were continued every other day after tumor cell injection for 12 days. For NK cell depletion, 50 μl of rabbit anti-mouse/rat asialo GM1 polyclonal Ab (Cedarslane Laboratories, Ontario, CA) was injected i.p. 1 day before tumor cell injection. Mice similarly injected with normal rabbit serum were used as controls. After tumor cell inoculation, Ab injections were continued twice per week for 2 wk. T and NK cell depletion was confirmed in lymph nodes and spleens 1 day before tumor challenge (for T cells) or on the same day of tumor cell challenge (for NK cells) by flow cytometry using relevant primary Abs followed by biotinylated secondary Abs. FACS analysis showed that >99% of the relevant population of T cells or NK cells were depleted in mice treated with anti-CD4, anti-CD8, and anti-asialo GM1. In contrast, mice treated with isotype controls displayed T lymphocyte profiles similar to the profiles of untreated mice (data not shown).

IL-21 ELISA

Overnight supernatants from 10^6 B16F1-IL-21, B16F1-GFP, MethA-IL-21, and MethA-GFP tumor cells were assayed for IL-21 levels by ELISA. In brief, murine IL-21RmIgG2aFc fusion protein (Wyeth Research) was used as coating Ab, and anti-mouse IL-21 (R&D Systems, Minneapolis, MN) was used as capture Ab. Purified mIL-21 (Wyeth Research) (4) was used as control. The detection limit for IL-21 is 12.5 ng/ml.

IL-21R mRNA expression detected by TaqMan

RNA was isolated from different tumor cell lines according to the manufacturer's instructions (Promega, Madison, WI). mRNA extracted from splenocytes that had been activated with plate-bound anti-CD3 (1 $\mu\text{g}/\text{ml}$) and anti-CD28 (1 $\mu\text{g}/\text{ml}$) mAbs (BD PharMingen) for 24 h was used for positive control. Purified RNA was treated with DNase (Ambion, Austin, TX) and adjusted to a concentration of 50 ng/ μl before mRNA analysis by quantitative TaqMan PCR analysis. IL-21R and cyclophilin-specific primer pairs and probes were designed using PrimerExpress software and were prepared by Wyeth Research (primers: 5'-GCCTTCTCAGGACGC TATGAT-3' and 5'-CCCTACAGCACGTAGTTGGA-3' and probe TC CTGGGACTCAGCTTATGACGAAAC). Standard curves for each gene were generated with RNA from known IL-21R-expressing cells. mRNA expression in control and transduced cell lines was normalized based on cyclophilin expression in each cell line, and the results are presented as relative units of mRNA.

Proliferation assay

Splenocytes (2×10^5 cells/well) from either C57BL/6 or BALB/c mice were stimulated with various concentrations of syngeneic irradiated tumor cells that expressed either GFP or IL-21 in 96-well plates. [³H]Thymidine at 1 $\mu\text{Ci}/\text{well}$ (PerkinElmer, Boston, MA) was added during the last 6 h of culture. After harvesting the supernatant onto glass fiber filter mats, [³H]Thymidine incorporation was determined by liquid scintillation counting.

ELISPOT assay

TRP-2-specific T cell responses were determined by the IFN- γ ELISPOT kit (R&D Systems) following manufacturer's instructions. Splenocytes

(2×10^5 – 4×10^5) in 200 μl of medium containing 20 U/ml murine IL-2 (BD PharMingen) were placed in each well in the presence of 5 $\mu\text{g}/\text{ml}$ specific TRP-2 peptides (9) or nonspecific OVA peptides. The plate was incubated for 24 h at 37°C in a CO₂ incubator. Plates were then incubated overnight at 4°C with detection Ab, followed by a 2-hr incubation with streptavidin-alkaline phosphatase conjugate. Spots were visualized with S-bromo-4 chloro-3' indolylphosphate p-toluidine salt/nitro blue tetrazolium chloride alkaline phosphatase substrate (R&D Systems). Plates were washed with tap water and air dried, and spots were counted with a stereomicroscope and recalculated to 10^6 cells with background spots subtracted. Generally, <10 spots/well were detected when OVA peptide was used as Ag.

In vitro restimulation of splenocytes from tumor cell-inoculated mice

Tumor peptide-specific T cell lines were generated as described elsewhere (13). In brief, mice were inoculated with either B16F1-GFP or B16F1-IL-21 cells. After 8–11 days, splenocytes were harvested and cultured with 5 $\mu\text{g}/\text{ml}$ TRP-2 peptide (9). On the third day of culture, 20 U/ml IL-2 (BD PharMingen) was added to each culture. After 5 days, cells were used for ⁵¹Cr release assay.

CTL assay

Cytotoxicity against targets was quantified using a 4-h ⁵¹Cr release assay. RMA-S cells (generously provided by Dr. K. Karre, Karolinska Institute, Stockholm, Sweden) were pulsed with TRP-2 peptide at 10 $\mu\text{g}/\text{ml}$ and labeled with Na₂⁵¹CrO₄ (PerkinElmer) for 1 h at 37°C. After washing, ⁵¹Cr-labeled target cells were incubated with T cell lines generated from C57BL/6 mice injected with tumor cells described earlier at different E:T ratios in 96 round-bottom plates. After a 4-h incubation at 37°C, supernatants were collected and radioactivity was detected in a scintillation counter (Wallac, Turku, Finland). Percent-specific lysis was calculated as $100 \times [(\text{release by CTL} - \text{spontaneous release}) / (\text{maximal release} - \text{spontaneous release})]$. Maximal release was determined by the addition of 1% Triton X-100. Spontaneous release in the absence of CTL was generally <15% of maximal release.

Detection of tumor Ag-specific CD8⁺ cell using tetramers

APC-conjugated MHC K^b tetramer complexes, as described elsewhere, (10) were purchased from Beckman Coulter (San Diego, CA). Draining lymph node cells from naive mice and mice injected with B16F1-IL-21 or B16F1-GFP tumor cells were stained with APC-labeled tetramers, FITC-labeled anti-CD3 (BD PharMingen), and PE-labeled anti-CD8 (BD PharMingen). The percentage of tetramer-positive cells was gated on CD8⁺ and CD3⁺ double-positive populations.

Prophylactic and therapeutic treatment of tumor

For prophylactic treatment, C57BL/6 mice were first vaccinated with 2×10^5 of irradiated (4000 rad) B16F1-GFP or B16F1-IL-21 tumor cells once or twice (1 wk apart) into the left flank. One week after vaccination, mice were challenged with 10^5 B16F1 tumor cells into the right flank. For therapeutic treatment, mice were first challenged with 10^5 of B16F1 cells on the back. One day or later, as indicated, treatment was initiated by injecting 10^6 of irradiated B16F1-IL-21 cells s.c. into the left flank.

Results

IL-21-transduced B16F1 and MethA cells secrete biologically functional IL-21

B16F1 melanoma and MethA fibrosarcoma tumor cells were transduced to express GFP plus IL-21 (B16F1/MethA-IL-21) or GFP (B16F1/MethA-GFP), respectively. GFP-positive cells were sorted and expanded, and IL-21 levels secreted by these cell lines were determined by IL-21 ELISA. B16F1-IL-21 and MethA-IL-21 cells, but not B16F1-GFP or MethA-GFP tumor cells, secreted a substantial amount of IL-21 in overnight cultures (Fig. 1*A*). To determine whether the IL-21 cytokine secreted by the transduced cells was biologically functional, irradiated IL-21 or GFP-expressing tumor cells were used to stimulate naive syngeneic splenocytes from C56BL/6 or BALB/c mice in the presence of suboptimal amounts of anti-CD3 and anti-CD28. B16F1-IL-21 enhanced naive splenocyte proliferation when compared with control GFP-expressing cells at all concentrations tested (Fig. 1*B*). Similar results

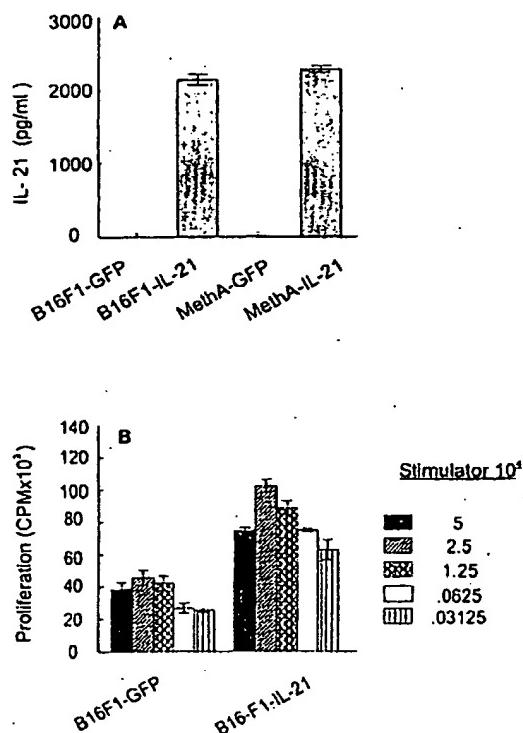


FIGURE 1. IL-21 secreted by IL-21-transduced B16F1 and MethA cells is biologically functional. *A*, Levels of IL-21 secretion by transduced tumor cells. Overnight supernatants from 10^6 of B16F1-IL-21, B16F1-GFP, MethA-IL-21, or MethA-GFP tumor cells were assayed by ELISA as described in Materials and Methods. *B*, Biological activity of IL-21 secreted by transduced tumor cells. Naive splenocytes from C57BL/6 mice were stimulated for 72 h with the indicated concentrations of irradiated syngeneic-transduced tumor cells. The cultures were supplemented with suboptimal amounts of anti-CD3 and anti-CD28 mAb in 96-well plates. [3 H]Thymidine was added during the last 6 h of culture.

were obtained with MethA-IL-21 cells (data not shown). These results suggest that IL-21 secreted by transduced tumor cells is biologically functional.

IL-21 does not affect tumor cell growth in vitro, but inhibits tumor formation in vivo

The in vitro growth kinetics of IL-21-producing tumor cells were very similar to that of the GFP-expressing control tumor cells (data not shown). This indicates that IL-21 does not have any apparent effect on the in vitro growth characteristics of transduced tumor cells. The unresponsiveness of tumor cells to IL-21 was further confirmed with the lack of IL-21R expression by tumor cells in real time PCR analysis. IL-21R expression was detected in anti-CD3- and anti-CD28-activated splenocytes, but not in any of the transduced tumor cell lines (Fig. 2*A*).

To assess the effect of paracrine-secreted IL-21 on the immune system during tumor growth in vivo, B16F1-IL-21 or MethA-IL-21 tumor cells were inoculated i.d. into the flank of syngeneic mice. B16F1 tumor cells are poorly immunogenic in that previous vaccination with irradiated wild-type tumor cells only protects 20% of vaccinated mice against subsequent live B16F1 challenge (14). In contrast, MethA, a methy-cholanthen-induced fibrosarcoma, is an immunogenic tumor model in which vaccination with irradiated MethA cells leads to almost 100% protection against

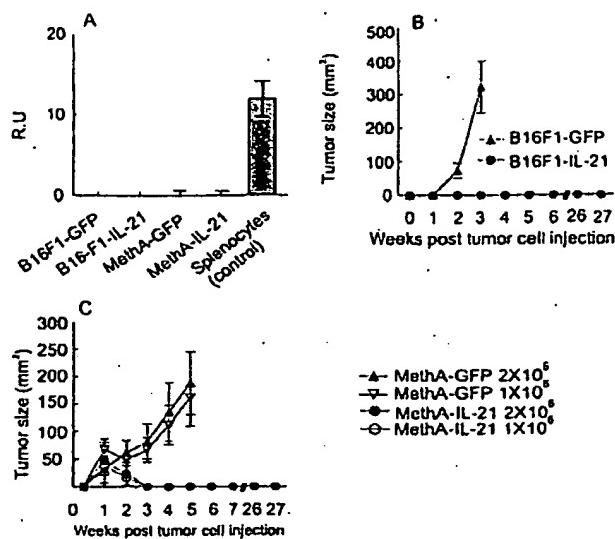


FIGURE 2. IL-21R expression by tumor cells and their in vivo growth characteristics. *A*, RNA was extracted from transduced tumor cells; cyclophilin and IL-21R mRNA present in the tumor cells were determined by TaqMan PCR. Expression of IL-21R mRNA in the transduced cells was normalized to cyclophilin values and expressed as relative units. RNA from anti-CD3 and anti-CD28 mAb-activated C57BL/6 splenocytes was used as positive control. *B*, Tumor growth in C57BL/6 mice that were injected with 10^5 of B16F1-IL-21 or GFP cells. *C*, Tumor growth in BALB/c mice that were injected with either 1×10^6 or 2×10^6 of MethA-IL-21 or MethA-GFP cells. Statistically significant difference ($p < 0.0002$) between tumor sizes in IL-21 and control tumor cell-injected mice was observed.

subsequent live MethA challenge (data not shown). Of interest, the only cytokines that have been reported to prevent B16F1 tumor formation in mice are IL-2 and IL-10 (14, 15).

In our experiments there was no tumor formation in any of the C57BL/6 mice inoculated with B16F1-IL-21 tumor cells for >27 wk after tumor injection (Fig. 2*B*). By contrast, all C57BL/6 mice bearing B16F1-GFP cells grew tumors starting as early as day 9. Control tumors increased rapidly in size, and these mice had to be sacrificed 2–3 wk after tumor cell inoculation because of a heavy tumor burden. In the MethA model, small but palpable tumor masses were detected 1 wk after tumor inoculation with either MethA-IL-21 or MethA-GFP cells in BALB/c mice (Fig. 2*C*). However, MethA-IL-21 tumors gradually reduced in size starting from wk 2 (day 11) and eventually regressed completely in 100% of mice, whereas 80% of control MethA-GFP tumors continued to grow in size until the mice were sacrificed. These results reveal the potency of IL-21 to trigger immune responses that lead to eradication of both immunogenic and nonimmunogenic tumors.

IL-21-induced anti-tumor response requires the presence of its cognate receptor (IL-21R)

The homology of IL-21 and IL-21R with other cytokines and cytokine receptors raises the question whether IL-21-induced anti-tumor responses require the interaction of IL-21 with its cognate receptor IL-21R. To address this question, B16F1-IL-21 cells were injected into the flank of IL-21R^{-/-} mice (4) or control C57BL/6 mice. IL-21-expressing B16F1 tumors grew out in 100% of IL-21R^{-/-} mice, but not in the control mice (Fig. 3). Thus, cognate interaction between IL-21 and IL-21R is crucial for the development of immune responses that lead to the rejection of IL-21-secreting tumor cells.

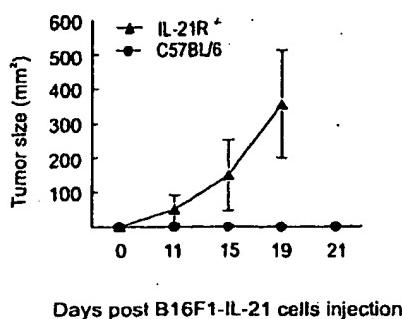


FIGURE 3. B16F1-IL-21 tumor growth in IL-21R^{-/-} mice. B16F1-IL-21 cells (10^5 /mouse) were injected into either IL-21R^{-/-} or normal C57BL/6 naive mice. Tumor size was monitored twice weekly. Significant difference ($p < 0.02$) between B16F1-IL-21-injected IL-21R^{-/-} mice and control C57BL/6 mice was observed.

IL-21 requires both innate and adaptive immunity in tumor rejection

Studies with genetically modified tumor cells have highlighted the participation of several types of cells, including neutrophils, eosinophils, mast cells, lymphocytes, and NK cells in tumor rejection (15–20). To determine the relative roles of lymphocytes in the rejection of IL-21-transduced tumor cells, equal numbers (10^5) of B16F1-IL-21 or control B16F1-GFP cells were injected into T and B cell-deficient (SCID) mice. Both B16F1-IL-21 and B16F1-GFP cells showed similar growth kinetics in SCID mice (Fig. 4A), indicating that lymphocytes (B and/or T cells) are required for IL-21-mediated tumor rejection. The indispensable role of T cells was confirmed in experiments with C57BL/6 nude/nude mice. All nude mice inoculated with B16F1-IL-21 cells developed tumors, albeit with slower growth kinetics (Fig. 4B).

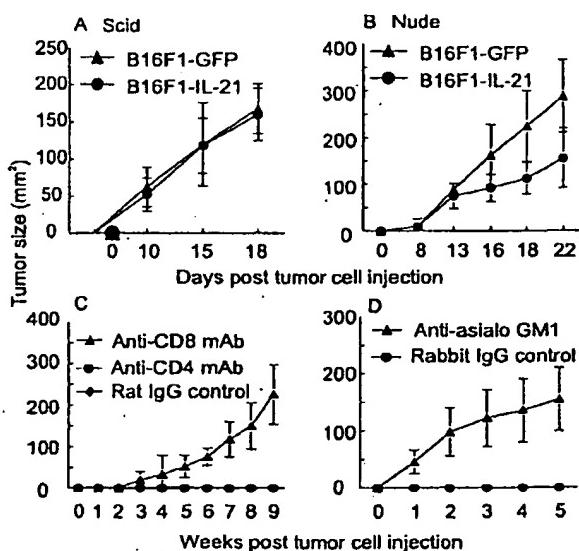


FIGURE 4. Growth of transduced B16F1 cells in immunodeficient and lymphocyte subpopulation-depleted mice. B16F1-IL-21 and B16F1-GFP tumor cells (10^5 /mouse) were injected into C57BL/6 SCID mice (A) C57BL/6 nude mice (B) CD4⁺ or CD8⁺ T cell-depleted mice ($p < 0.005$, $n = 9$) (C), or NK cell-depleted mice ($p < 0.001$, $n = 9$) (D). Rat IgG or rabbit IgG was used as isotype controls for T and NK cell depletion, respectively. Tumor size was monitored twice per week as described in Fig. 3.

To further dissect which T cell subset(s) is (are) important for the IL-21-induced effect, we performed in vivo depletion of lymphocyte subpopulations by administering anti-CD4 or anti-CD8 mAbs. B16F1-IL-21 tumors did not grow in CD4⁺ T cell-depleted mice and in control rat IgG-treated mice (Fig. 4C). However, palpable tumors grew out in 7 of 10 CD8⁺ T cell-depleted mice, suggesting that CD8⁺, but not CD4⁺, T cells are necessary for the IL-21-induced anti-tumor response (Fig. 4C). Of interest, the growth of B16F1-IL-21 in CD8⁺ T cell-depleted mice was significantly delayed, suggesting that cells other than CD8⁺ T cells may be responsible for the suppression of early phase tumor growth.

Accumulated experimental evidence supports the role of NK cells as a first line of defense in promoting anti-tumor immunity (21–23). We examined this possibility by injecting equal numbers of B16F1-IL-21 tumor cells into anti-asialo GM1-treated mice or control C57BL/6 mice. As expected, no tumor formation was observed in control C57BL/6 mice, whereas all of the anti-asialo GM1-treated mice grew tumors as early as 2 wk after B16F1-IL-21 tumor cell inoculation (Fig. 4D). Of note, we also used anti-NK1.1 Ab for NK cell depletion and observed the same results as in anti-asialo GM1-treated mice. This further confirms that NK cells are required for the rejection of B16F1-IL-21 tumors. Taken together, these results indicate that IL-21 activates NK and CD8⁺ T cells and that tumor eradication requires the participation of both innate and adaptive immunity.

IL-21 supports the generation of IFN- γ -secreting tumor Ag-specific T cells and enhances tumor Ag-specific CTL activity

We have used the B16F1 tumor-specific TRP-2 peptide to evaluate the effect of IL-21 on T cell responses in vivo (9, 10). The presence of TRP-2-specific CD8⁺ T cells in tumor cell-injected mice was determined with tetramers containing TRP-2 peptides. Single cell suspensions from draining lymph nodes of naive (Fig. 5A) B16F1-GFP- (Fig. 5B) or B16F1-IL-21- (Fig. 5C) injected mice were stained with TRP-2 tetramer. Compared with B16F1-GFP-inoculated mice, B16F1-IL-21 cell-injected mice possessed more than a 2-fold increase in the number of tumor-specific CD8⁺ T cells. As expected, cells from naive mice stained at a background level of tetramer. To examine whether these T cells are functional, splenocytes from mice injected with either IL-21 or GFP-expressing B16F1 cells were stimulated with TRP-2 peptide or OVA control peptide in an IFN- γ ELISPOT assay. After the background with OVA peptide was subtracted, the number of IFN- γ -producing cells in B16F1-IL-21-injected mice was 3-fold higher than that of B16F1-GFP-injected mice (Fig. 5D). Although TNF- α and IL-10 ELISPOT were performed similarly, no significant differences in spot-forming units were detected between B16F1-GFP and B16F1-IL-21 tumor cell-injected mice (data not shown). To further characterize IL-21-mediated anti-tumor T cell responses, splenocytes from either IL-21 or GFP-expressing tumor-injected mice were first stimulated with TRP-2 peptide in vitro before CTL assays. Splenocytes from B16F1-IL-21-injected mice had enhanced cytolytic activity toward TRP-2 peptide-pulsed RMA-S cells compared with splenocytes from GFP-expressing tumor-bearing mice at all E:T ratios (Fig. 5E). These results indicate that IL-21 enhances the development of tumor Ag-specific cytolytic T cell responses. Of note, TRP-2 is one of the Ags shared between B16F1 tumor cells and normal melanocytes. Thus, CTLs detected in this study are actually autoreactive T cells. Indeed, 10–20% of C57BL/6 mice developed local hair and skin depigmentation at the injection site of B16F1-IL-21 tumor cells, but not at the injection site of control cells (Fig. 5F).

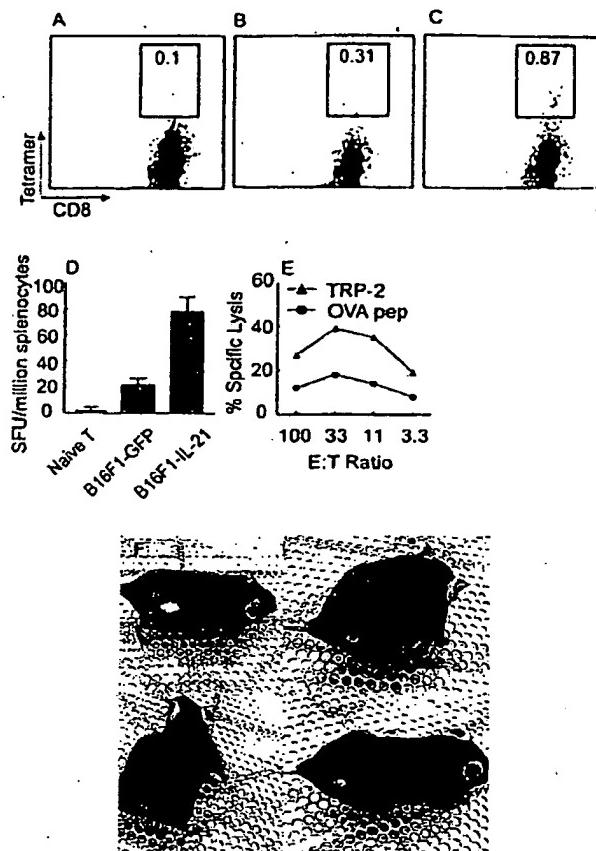


FIGURE 5. TRP-2-specific T cell responses in B16F1-IL-21-injected mice. Draining lymph nodes from naive mice (*A*), mice injected with B16F1-GFP (*B*), or B16F1-IL-21 cells (*C*) 8 days earlier were harvested. Single cell suspension was stained with TPR-2 tetramer (APC), anti-CD8 mAb (PE) and anti-CD3 Ab. Lymph node cells from naive mice were used as control. Results shown are the percentage of tetramer-positive cells gated on CD3⁺ and CD8⁺ cells. *D*, Equal numbers of splenocytes ($2-4 \times 10^6$) from mice in *A-C* were stimulated with 5 $\mu\text{g}/\text{ml}$ TRP-2 or OVA control peptide in the presence of 20 U of IL-2 in an ELISPOT plate precoated with anti-IFN- γ Ab. After 24 h, the plate was developed, and spot-forming units were counted. Results are expressed as the number of spot-forming units/million splenocytes, with the background to OVA peptide being subtracted (spot-forming units/million splenocytes). *E*, Cytolytic activity of splenocytes from B16F1-IL-21 or control B16F1-GFP-injected mice were tested against RMA-S cells pulsed with TRP-2, with background against OVA peptide (control) subtracted. Cytolytic activity was measured by standard 4-hr Cr⁵¹ release assay. *F*, Hair and skin depigmentation at the sites of B16F1-IL-21 tumor injection.

IL-21-induced anti-tumor response is independent of IFN- γ , IL-12, IL-4, and IL-10, but requires psp

It is well established now that IFN- γ and IL-12 are the cytokines primarily involved in activating immune cells against tumors, both in murine models and in humans (24–26). To definitively determine whether these Th1/Th2 cytokines actively participate in IL-21-mediated anti-tumor response, equal numbers of B16F1-IL-21 or B16F1-GFP tumor cells were injected into C57BL/6 mice deficient of IFN- γ , IL-12, IL-10, or IL-4. Interestingly, none of the aforementioned cytokine-deficient mice formed tumors after B16F1-IL-21 cell injection (Fig. 6*A–D*). However, B16F1-GFP control cells grew tumors in those mice. The observation that tu-

mor rejection is IFN- γ independent may have clinical importance, because production of IFN- γ during an overwhelming anti-tumor response has been accompanied by severe side effects (27, 28).

Besides cytokines, NK and CD8⁺ T cells can also reject tumors via a psp-mediated cytotoxic mechanism (29, 30). To investigate whether IL-21-induced anti-tumor response requires the psp-mediated pathway, B16F1- or GFP-expressing tumor cells were injected into psp^{−/−} mice. In these experiments, 100 and 80% of the psp^{−/−} mice injected with B16F1-GFP or B16F1-IL-21 cells, respectively, developed tumors (Fig. 6*F*). Interestingly, B16F1-IL-21 cell-injected mice showed delayed tumor growth compared with control-injected mice (Fig. 6*E*). The growth of B16F1-IL-21 cells in psp^{−/−} mice indicates that IL-21-induced anti-tumor response involves psp-mediated cytotoxicity.

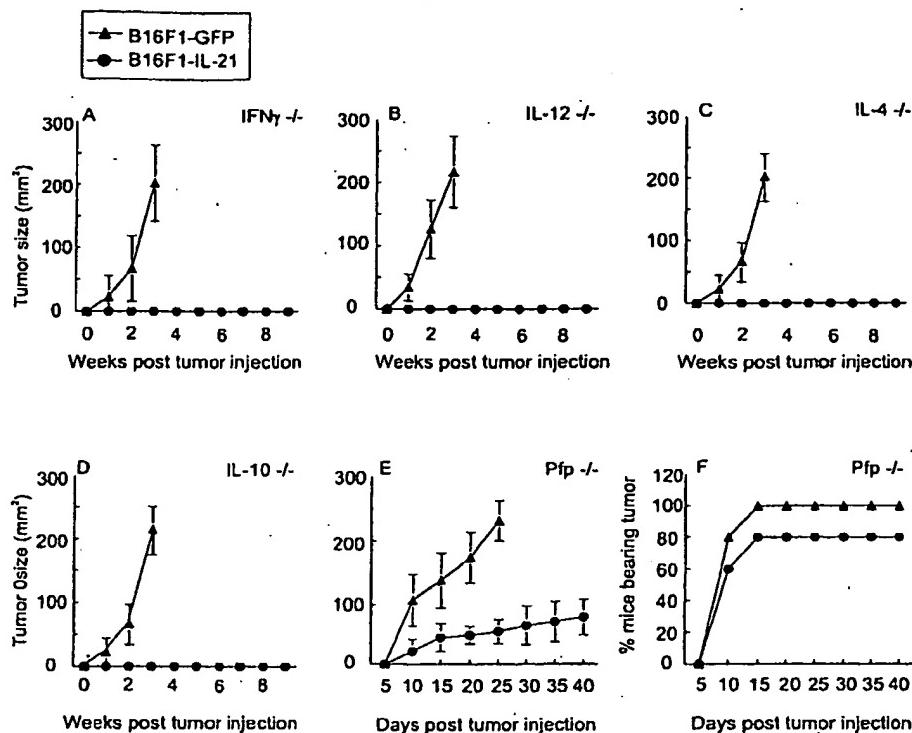
IL-21 mediates a potent prophylactic and therapeutic effect

In preliminary vaccination experiments, we observed that 30–40% of mice that rejected primary B16F1-IL-21 tumors were also protected from subsequent B16F1 tumor challenge when it was administered 3 wk later in the opposite flank. Nevertheless, none of these mice was protected when challenged with bladder carcinoma MB49 tumor cells (data not shown). Therefore, we sought to evaluate the role of IL-21 in prophylactic and therapeutic treatment of tumors. For prophylactic treatment, mice were vaccinated once or twice with irradiated B16F1-IL-21 or B16F1-GFP 1 wk before B16F1 challenge. Mice vaccinated with irradiated B16F1-IL-21 showed substantially delayed tumor growth when compared with the mice that received irradiated B16F1-GFP cells (Fig. 7*A*). Interestingly, irradiated B16F1-IL-21 vaccination followed by boosting conferred better protection than a single vaccination (Fig. 7*A*). This suggests that IL-21 can induce effective anti-tumor memory response against subsequent challenge of tumor cells. To evaluate the therapeutic potential of IL-21, irradiated B16F1-IL-21 cells were administrated s.c. 1, 3, and 5 days after mice were challenged s.c. with 10^5 live B16F1 cells. Injection of irradiated B16F1-GFP cells did not show any therapeutic effect upon B16F1 cell challenge (data not shown). However, significant delay in tumor growth was observed in mice treated with irradiated B16F1-IL-21 cells, and 70% of tumor-bearing mice treated with three doses of irradiated B16F1-IL-21 cells rejected tumors completely (Fig. 7*B*). Of note, all of the mice that were cleared of tumors also developed autoimmune depigmentation of the skin in the surrounding as well as more distant areas (data not shown).

Discussion

In this study we have examined the *in vivo* biology of IL-21 using tumor models. We validate previous *in vitro* observations by showing that IL-21 has a very potent *in vivo* effect on both NK and CD8⁺ T cells that results in eradication of both immunogenic and nonimmunogenic tumors. Of interest, IL-21-mediated tumor rejection is independent of major Th1 and Th2 cytokines, i.e., IFN- γ , IL-12, IL-4, and IL-10, but requires the psp-mediated cytotoxic pathway. Finally, we demonstrate that, when used as prophylactic or therapeutic vaccine, IL-21 leads to 50 and 70% tumor rejection, respectively.

The rapid and definitive elimination of IL-21-transduced B16 melanoma tumor cells observed in our studies is in clear contrast with data obtained in other cytokine gene vaccination models, underscoring a unique role of IL-21 in modulating the immune system. Mice immunized with IL-4-, IL-5-, IL-6-, IL-12-, IFN- γ , TNF- α -, or GM-CSF-transduced B16 vaccines, as reported previously, displayed moderate delays in tumor formation. However, eventually all mice succumbed to lethal tumors (14, 31). Of note,



GM-CSF-, IL-5-, IL-6-, and TNF- α -expressing cells caused significant side effects (14, 31). To date, only IL-2 and IL-10 have been reported to induce complete regression of transduced B16 tumors *in vivo* (14, 15). In our study, syngeneic mice injected with B16F1-IL-21 or MeihA-IL-21 tumor cells did not develop any

clinically overt tumor for a period of >41 wk after tumor inoculation.

Interestingly, mice injected with B16F1-IL-21 tumor cells developed hair and skin depigmentation around the injection sites. Presumably, autoimmunity against normal mouse melanocytes was induced through (a) common epitope(s) shared with B16 tumor cells in the close proximity of tumor injection sites (9, 10, 31). To our knowledge, IL-21 is the only cytokine that has caused local depigmentation upon injection of cytokine gene-modified B16F1 tumor cells, thus validating its unequivocal ability in activating potent immune responses. However, the fact that flow cytometric analysis of spleen and lymph node cells removed from immunized mice did not show any major changes in cell population (data not shown), suggests that paracrine secretion of IL-21 modulates the immune system without causing overt systemic side effects.

Despite the apparent redundancy in the IL-21R signaling pathway with other γ c-dependent cytokine receptors, B16F1-IL-21 tumor grew out in IL-21R $^{-/-}$ mice, but not in CS7BL/6 mice. This suggests that IL-21 can act only through its cognate receptor IL-21R. Additionally, this also implies that IL-21 expressed by tumor cells is functioning through cells of host origin. Of note, the outgrowth of B16F1-IL-21 tumors in IL-21R $^{-/-}$ mice should not be attributed to an intrinsic defect in T and NK cells, as IL-21R $^{-/-}$ mice have normal NK and T cell development, and those cells respond to cytokines other than IL-21 (4).

IL-21 promotes innate immune responses by enhancing cytotoxicity and IFN- γ production of NK cells (1, 4). B16F1-IL-21 tumors grew out in mice depleted of NK cells, demonstrating that IL-21 is acting on NK cells to potentiate tumor rejection. In these mice tumors grow out quickly after inoculation, consistent with an early role for NK cells in tumor rejection, which is likely to account for the significantly delayed B16F1-IL-21 tumor growth in CD8 $^{+}$ T cell-depleted mice. IL-21 may directly enhance anti-

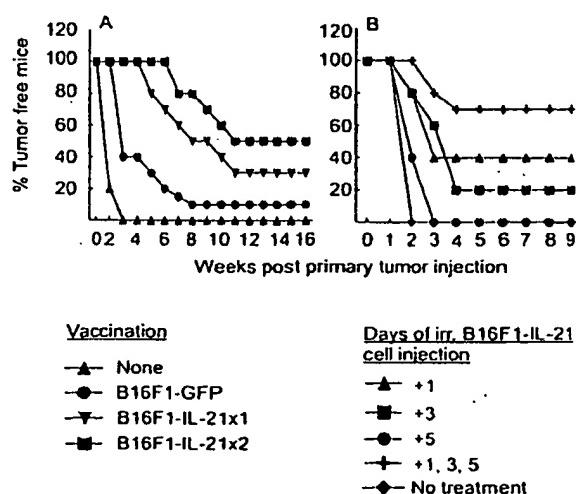


FIGURE 7. Prophylactic and therapeutic anti-tumor response of IL-21. *A*, C57BL/6 mice were first vaccinated with 2×10^5 irradiated (4000 rad) B16F1-GFP or B16F1-IL-21 tumor cells once or twice (1 wk apart) into the left flank. One week after vaccination, mice were challenged with 10^5 B16F1 tumor cells into the right flank. For therapeutic treatment, C57BL/6 mice were first challenged with 10^5 B16F1 cells in PBS on the back. One day or later, as indicated, treatment was initiated by injecting 10^6 of irradiated B16F1-IL-21 cells s.c. into the left flank (*B*).

tumor lytic activity of NK cells, and/or NK cell activation may enhance macrophage cytotoxicity and facilitate tumor Ag processing and presentation to T cells (32, 33). Thus, NK cells keep tumor growth under control, during which time the adaptive immune response is initiated. Although NK cells are present in both SCID and nude mice (34), there is no significant delay in tumor formation in those mice. However, these mice also lack CD4⁺ T cells and NKT cells, in contrast to the CD8-depleted mice. Although CD4⁺ T cells are not required for rejection of B16F1-IL21 tumors, they may contribute to tumor rejection in the absence of CD8⁺ cells. Alternatively, the explanation may lie in NKT cells; NKT cells are a component of NK cells that express both NK markers and TCR. They can be promptly activated to release cytokines that may contribute to NK cell activation. Thus, the possible absence of NKT cells in SCID and nude mice (35, 36) may result in rapid initial tumor growth caused by lack of activated NK cells.

IL-21 promotes Ag-specific (adaptive) anti-tumor responses as evidenced by the restoration of IL-21-expressing tumor growth in SCID and nude mice. Both CD4⁺ and CD8⁺ T cells have been described to be important for the induction of tumor regression and the development of protective immunity (17, 18, 37, 38). At first glance, the finding that CD8⁺, but not CD4⁺, T cells are required for the anti-tumor response of IL-21 is not in agreement with the doctrine that CD4⁺ T helper cells are required for activation of naive CD8⁺ T cells (39). However, recent studies have demonstrated that *in vivo* elimination of CD4⁺ T cells may actually enhance the anti-tumor effect in cytokine gene therapies. This is accomplished by either skewing the cytokine milieu to Th1 phenotype or removing CD4⁺CD25⁺ T regulatory cells (10, 31). Because activated CD4⁺ T cells are a major source of IL-21 (1), we further speculate that IL-21 released by B16F1-IL-21 may replace the help that is normally provided by CD4⁺ T cells. Thus, IL-21 may act on CD8⁺ T cells directly by lowering the threshold of costimulation necessary for their activation and/or, indirectly, by augmenting Ag presentation of the IL-21R-bearing APCs (40–42). Although B16F1-GFP control tumor cells elicited some level of tumor-specific CTL responses, these responses were not sufficient for tumor clearance. In this regard, as our experiments with tetramers demonstrate, IL-21 potentiates the expansion of Ag-specific CD8⁺ T cells, which ultimately leads to tumor elimination.

IL-21 also enhances IFN- γ production by both NK and CD8⁺ T cells as demonstrated by our results and others (1, 4). IFN- γ is a pleiotropic cytokine that can act on both tumor cells and host immunity (32, 43). IFN- γ directly inhibits proliferation of some tumor cells *in vitro* (44) and indirectly inhibits tumor growth *in vivo* by suppressing tumor angiogenesis (45, 46). Nevertheless, IFN- γ and other cytokines (i.e., IL-12, IL-4, IL-10) are not required for IL-21-induced anti-tumor responses. Thus, it is likely that IL-21 acts either directly or through mediators other than IFN- γ (and IL-12, IL-4, IL-10) to potentiate the expansion of NK and tumor-specific CD8⁺ T cells and to enhance their cytotoxic activity via the pfp (and/or Fas/Fas ligand) pathway. Indeed, the fact that B16F1-IL-21 tumors grew in pfp^{-/-} mice strongly suggests that the pfp-mediated cytotoxic pathway is indispensable for IL-21-induced anti-tumor responses. Of note, the observed reduced growth rate of B16F1-IL-21 tumors in pfp^{-/-} mice, compared with B16F1-GFP tumors, indicates that other factors induced by IL-21 may also be involved in tumor surveillance.

Finally, we demonstrate in this study that IL-21 alone has the unique potential to prevent or cure poorly immunogenic B16 melanoma tumors. It has been shown previously that GM-CSF-based cancer vaccines protect mice from developing B16 tumors when given prophylactically (47). However, therapeutic GM-CSF vaccines alone have little to no effect on the eradication of pre-existing

tumors, and combination treatment (e.g., with anti-cytotoxic T lymphocyte Ag, CTLA-4 Ab) (48) is required. In our study, irradiated B16F1-IL-21 vaccines protected 50% of mice from subsequent tumors and cured 70% of mice with pre-existing tumors. It is noteworthy that, although historically it has been easier to prevent than cure established tumors, in our study prophylactic IL-21 vaccines are less potent than therapeutic vaccines. The better efficacy of IL-21 in therapeutic vaccines may be because of the effects of IL-21 on both NK and CD8⁺ cells. IL-21 has been shown to enhance NK effector function, but IL-21 does not support expansion of NK cells (4). Thus, IL-21 may be more potent once NK cells have been activated and have begun to expand. This would allow for better NK cytotoxicity, which may also result in enhanced tumor Ag presentation. In addition, IL-21 can potentiate CTL function, and our data suggest that the effects of IL-21 are more potent after priming of the CTL compartment. Nevertheless, in the prophylactic settings, IL-21 is not available to enhance the effector function of NK or CD8⁺ T cells upon initial tumor challenge, as IL-21 is primarily produced by activated T cells. Of interest, all the mice that were cleared of tumors in the therapeutic setting (but not in the prophylactic setting) also developed autoimmune depigmentation (data not shown); a fact that has been correlated both in murine models and human trials, with the establishment of a potent anti-tumor response (9, 49). To our knowledge IL-21 is the first cytokine so far that has been shown to induce autoimmune depigmentation and thus vigorous anti-tumor response. By the time we submitted this manuscript, Ugai et al. (50) published similar findings in murine colon carcinoma models. Their report also highlighted the importance of IL-21 in inducing effective antitumor responses through NK and CD8⁺ T cells. In conclusion, the unique antitumor effect and the safety profile of IL-21 described in this report opens new possibilities for immunotherapy in cancer.

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MACROPHAGE FACTORS WHICH ENHANCE THE MIXED LEUKOCYTE REACTION INITIATED BY DENDRITIC CELLS¹

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Stimulator cells for the allogeneic MLR are dendritic cells but not macrophages (Mφ). Mφ, however, enhance the MLR initiated by relatively low doses of dendritic cells. The present report demonstrates that the enhancement of the MLR is mediated by two factors produced by Mφ. One is IL-1, inasmuch as it has a M_r ~15 kDa, and both partially purified IL-1 and rIL-1 also enhance the MLR. The other has been identified as granulocyte-macrophage (GM)-CSF. It had a M_r ~25 kDa, and is reproduced by rGM-CSF. Moreover, the MLR-enhancing activity of both the 25-kDa molecule and rGM-CSF have been neutralized by anti-GM-CSF antiserum. Both IL-1 and GM-CSF have autonomous enhancing activity, but they collaborate with each other in enhancing the MLR. Both factors act on DC to augment their stimulatory activity for allogeneic T lymphocytes.

We have studied the role of Mφ and DC in the immune responses, and reported that the Ag-presenting activity for primary and also for secondary immune responses as well as the stimulatory capacity for primary MLR of DC are incomparably higher than those of Mφ (1-3). Mφ, however, modulate the immune responses initiated by DC. An enhancing effect of Mφ is observed, especially when the number of DC is so small to trigger a strong response.

Recently, Koide et al. (4, 5) reported that accessory and stimulatory capacity of DC are augmented by IL-1 produced by Mφ. The results to be shown in this paper also support this. Furthermore, we report here concerning another factor which is also produced by Mφ and exert the effect not only autonomously but also synergistically with IL-1.

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⁴Abbreviations used in this paper: Mφ, macrophages; DC, dendritic cells; GM, granulocyte-macrophage; LAF, lymphocyte-activating factor; NMS, normal mouse serum; LC, Langerhans cells; PEC, peritoneal exudate cells.

MATERIALS AND METHODS

Mice. BALB/c, [BALB/c × DBA/2]F1 (CD2F1), C3H/HeSic, C57BL/6, and C57BL/10 mice raised in our breeding colony or purchased from Shizoku Agricultural Cooperative Association for Laboratory Animals, Shizuoka, were used at the age of 3 to 5 mo as the donor of Mφ, DC, and responder T cells. Thymocytes were prepared from 6- to 6-wk-old mice.

Cytokines and antibodies. Con A-stimulated spleen cell supernatant and murine rIFN-γ were used to stimulate Mφ. The former was prepared as described by Maruccci et al. (6) and the latter was provided by the Research Institute of Shionogi Pharmaceutical Co. Ltd., Osaka. Natural IL-1 was partially purified by HPLC gel filtration from the supernatant of protease peptone-elicited Mφ stimulated with latex particles (7). Human rIL-1α was donated by Dainippon Pharmaceutical Co. Ltd., Osaka (8). Murine rGM-CSF was partially purified from the medium of COS-1 cells transfected with plasmid DNA pHs mGM-CSF (9). Rabbit anti-GM-CSF antiserum was a gift of Immunex Co., Seattle (10). Culture supernatants of WEHI-3 cells was used as a crude IL-3 preparation. Mouse purified IL-3 was purchased from Genzyme Corporation, Boston, MA. mAb MRD8 (anti-In⁺), B21-2 (anti-la⁺), and 10.2.16 (anti-la⁺) were used as culture supernatant to deplete la-bearing cells with the aid of C (Low-Tox-M rabbit C; Cedarlane Laboratories, Ltd., Hornby, Ontario).

Mφ and Mφ supernatant. Mφ were prepared as adherent cells from PEC elicited by thioglycollate medium as described by Naito et al. (1). In order to prepare Mφ supernatant, 5 to 10×10^6 PEC in 10 ml and 1 to 2×10^6 in 1 ml were plated into 100-mm diameter plastic dishes (Falcon 3003; Oxnard, CA) and 24-well plates (A/S Nunc, Kamstrup, Roskilde), respectively. Adherent Mφ (half of initially plated PEC in number) were precultured for 4 to 5 days in the presence or absence of lymphokine in RPMI 1640 (Nissui Seiyaku Co. Ltd., Tokyo) supplemented with 5% FCS (Sterile Systems, Logan, UT), 2 mM l-glutamine, penicillin (100 U/ml), streptomycin (100 µg/ml), and 10 mM HEPES. After replacing the medium with modified Click's medium containing 0.5% NMS and 1 µg/ml indomethacin (Sigma Chemicals Co., St. Louis, Mo.) (1), Mφ supernatant was collected 24 h later. In most cases Mφ populations were pretreated with 20 µg/ml mitomycin C (Kyowa Hakko Co. Ltd., Tokyo) for 30 min at 37°C to prevent the growth of contaminating fibroblasts during the culture.

For obtaining the supernatant to be submitted to the gel filtration, Mφ were cultured in Click's medium without NMS for 24 h. The supernatant was concentrated 15- to 20-fold by Diaflo ultrafiltration membrane YM5 (Amicon Corporation, Lexington, MA), and dialyzed against 0.01 M PBS. Fractionation was performed by an HPLC gel filtration column (TSK-GEL, G3000SWG column; Toyo Soda Manufacturing Co. Ltd., Tokyo) equilibrated with 0.01 M PBS at a flow rate of 3 ml/min. The column was calibrated with BSA (67 kDa), OVA (43 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (20.1 kDa), and RNase (13.7 kDa).

Cells. DC were prepared from spleen cells according to the methods of Nussenzweig and Steinman (11) with a slight modification as described previously (1). Responder T cells were prepared by passing spleen cells successively through nylon wool and Sephadex G-10 (Pharmacia Fine Chemicals, Uppsala) columns (12, 13) followed by depletion of la-bearing cells with anti-la antibodies and C.

Allogeneic MLR. The MLR was performed as described (1) with a slight modification. Briefly, 3 to 5×10^6 responder T cells and graded numbers of DC were cultured in 0.2 ml of modified Click's medium in 96-well flat-bottomed microplates (Nunc). In the double chamber culture system, 18-mm diameter plates were used as lower chambers. The upper chamber was prepared by assembling one side of an acryl cylinder with a membrane filter (0.22 µm porosity; Millipore

Co., Bedford, MA). DC and 1.5×10^6 T cells were cultured in the upper chambers, and Mφ were placed in the lower chambers. One set of the double chamber cultures contained 1 ml of medium.

T cell proliferation was assessed by ^{3}H TdR (ICN Radiochemicals, Irvine, CA) incorporation during the last 6 to 12 h of culture.

Co-mitogenic assay. The activity of LAF was assayed as co-mitogenic activity for thymocytes with PHA (Difco Laboratories, Detroit, MI) or Con A (Pharmacia Fine Chemicals) as described by Hirayama et al. (7).

RESULTS

Enhancement of the allogenic MLR is mediated by soluble factors from Mφ. As shown in our previous report, Mφ, which are inadequate as the stimulator cells, enhance the MLR triggered by DC (1). Thus, the presence of appropriate number of Mφ makes a dose of 0.2% DC to be as effective as a dose of 2% DC used in the absence of Mφ.

Mφ need not contact directly either responder T cells and/or DC. In the experiment of Table I, the direct interaction between Mφ and the other cells was hampered by membrane filters, but the MLR to allogeneic DC in upper chambers was markedly enhanced when Mφ were placed in the lower chamber. This result also indicates that the enhancement of the MLR is mediated by soluble factor(s) released from Mφ.

The following experiments were conducted to identify the Mφ-derived factors and the mechanism for augmenting T cell proliferation. We prepared Mφ supernatant from four different Mφ populations, which were freshly

prepared or precultured in the presence or absence of rIFN-γ or Con A-stimulated spleen cell supernatant. MLR-enhancing activity and LAF activity were assessed (Table II). No significant difference in the MLR-enhancing activity was observed among the four different Mφ supernatant preparations. Concerning the LAF activity, however, the supernatant from freshly prepared Mφ was higher than those from other Mφ preparations. These results provided a clue that more than one factor might be involved in the augmentation of the MLR.

Therefore, Mφ-conditioning medium was fractionated by gel filtration and each fraction was assayed for MLR-enhancing activity and LAF activity (Fig. 1). The main peak of MLR-enhancing activity was observed in the fractions comprising 20- to 30-kDa molecules (Fig. 1A). On the other hand, LAF activity was detected in the fractions of around 15-kDa molecules (Fig. 1B). At a higher concentration (5 times higher than in the experiment of Fig. 1), a low but significant level of MLR-enhancing activity was also detected in fractions ranging from 13 to 20 kDa, which of course possessed high LAF activity (not shown). Even at such a high concentration, however, 20- to 30-kDa molecules manifested no LAF activity. In other experiments, we noticed that partially purified IL-1 prepared from proteose peptone-elicited Mφ was also able to augment the MLR to some extent (Table III), confirming the report of Koide et al. (4). Taken together, these results indicate that the augmentation of the MLR is mediated by at least two distinctive factors: one is most probably IL-1 and the other possibly more potent factor comprises molecules of about 25 kDa.

To test whether these factors collaborate to enhance the MLR, graded doses of semipurified IL-1 and unfractonated Mφ supernatant were added separately or together to the MLR (Table III). To obtain clear results, we selected Mφ supernatants which exhibited little LAF activity. Either IL-1 or the Mφ supernatant alone enhanced the MLR in a dose dependent manner, but the enhancement was about 4-fold. Much great enhancement was evident, when both IL-1 and Mφ supernatant were added, and these enhancing effects appeared synergistic rather than additive. On the other hand, the Mφ supernatant we used exerted no effect on thymocyte proliferation in the presence of PHA and therefore had little IL-1. Thus, IL-1 and a distinct 25-kDa factor synergize with each other to

TABLE I
Enhancing effect of Mφ does not require the direct cellular contact

Chambers ^a		MLR ^b (cpm × 10 ⁻³)	
Upper	Lower	Expt. 1	Expt. 2
T cells	None	2.9	0.9
T cells + 3 × 10 ⁶ DC	None	6.5	2.2
	Mφ from 10 ⁶ PEC	ND	22.8
	Mφ from 2 × 10 ⁶	180.0	100.2
	PEC		
T cells + 3.5 × 10 ⁶ (Expt. 1) or 6.7 × 10 ⁶ (Expt. 2) DC	None	70.8	152.4

^a Responder T cells (1.5×10^6) from B6 mice in experiment 1 or CD2F1 mice in experiment 2 were cultured with or without B10.BR DC in upper chambers. The indicated numbers of B10.BR Mφ, precultured with rIFN-γ at 12.5 U/ml for 7 days, were placed in lower chambers.

^b Figures are relative values, because incorporation of radioactivity was expressed as one of 0.1-ml aliquots of the culture in the upper chambers.

TABLE II
MLR-enhancing and LAF activities of the supernatant of freshly isolated or precultured Mφ cultures^c

Source of Mφ Culture Supernatant ^d	No. of cultured PEC needed	MLR (cpm × 10 ⁻³) with Dilution of Mφ Supernatant:			LAF Activity (cpm × 10 ⁻³) with Dilution of Mφ Supernatant:		
		1/2	1/4	1/8	1/2	1/4	1/8
Fresh	10 ⁶	51.5	9.4	2.0	80.8	69.6	20.1
	2 × 10 ⁶	78.5	17.5	3.3	92.6	42.5	26.7
Precultured	10 ⁶	56.8	11.5	3.0	20.8	40.6	11.8
	2 × 10 ⁶	45.3	9.4	3.9	9.5	7.8	9.1
Precultured with rIFN-γ	10 ⁶	58.3	12.4	1.7	25.2	13.0	18.2
	2 × 10 ⁶	63.8	15.4	3.0	12.5	15.8	14.8
Precultured with CS	10 ⁶	56.4	12.2	3.7	24.9	14.6	18.0
	2 × 10 ⁶	50.4	15.2	5.9	17.8	15.3	14.0
None ^e		2.6			10.9		

^c For MLR, CD2F1 responder T cells (4×10^6) and B10.BR DC (1.5×10^6) were cultured for 4 days. ^{3}H TdR incorporation of responder cells alone was 289 in the absence of Mφ supernatant, and 59 to 326 in its presence. LAF activity was assessed by using C3H/HeSic thymocytes (5×10^6 /culture) and PHA (1 µg/ml).

^d Four kinds of Mφ supernatant were prepared as described in the text.

^e MLR and LAF assays were performed in the absence of Mφ supernatant.

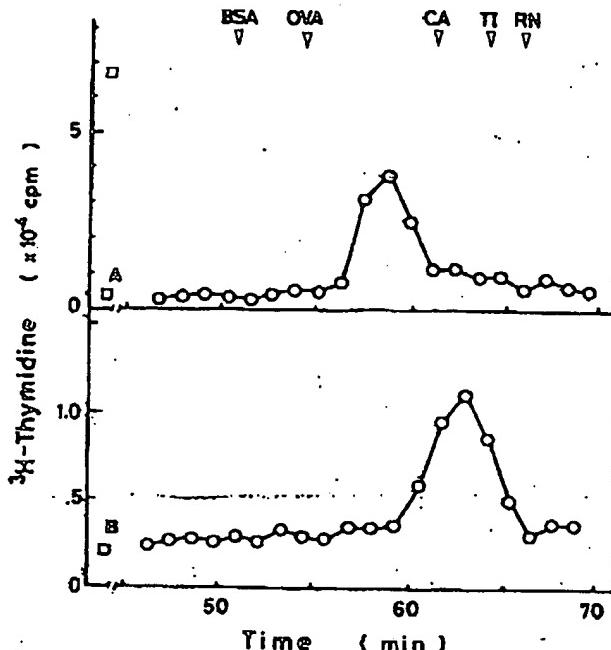


Figure 1. Gel filtration of Mφ supernatant for identifying fractions with MLR-enhancing activity and LAF activity. Culture supernatant of C3H/HeSk Mφ, precultured without lymphokines, were concentrated and fractionated by an HPLC TSK-GEL G3000SWG column equilibrated with 0.01 M PBS at a flow rate of 3 ml/min. The column was calibrated with BSA (67 kDa), OVA (43 kDa), carbonic anhydrase (CA; 30 kDa), trypsin inhibitor (TI; 20.1 kDa), and RNase (RN; 13.7 kDa). In A, BALB/c responder cells (5×10^6 /culture) and B10.BR DC (1.5×10^6 /culture) were cultured for 4 days in the presence (□) or absence (○) of the whole Mφ supernatant, or in the presence of each fraction (5% v/v; ○). In B, LAF activity of each fraction (12.5% v/v) was assessed using C3H thymocytes (5×10^6 /culture). Open square represents the reaction of thymocytes without any fractions of Mφ supernatant.

augment the MLR.

The 25-kDa factor is plausibly GM-CSF. Concerning the identity of the 25-kDa Mφ factor, we considered two candidates, GM-CSF (29 kDa) and IL-3 (28 kDa), based only on the M_r (14–16). First, the MLR-enhancing activity of murine rGM-CSF was tested (Fig. 2). Both the rGM-CSF and the 25-kDa factor significantly amplified the MLR. The MLR-enhancing activity of both rGM-CSF and 25-kDa factor was neutralized by anti-GM-CSF antiserum, suggesting that the 25-kDa molecule is most probably GM-CSF. In contrast to GM-CSF, neither WEHI-3 supernatant, which is a crude but active source of IL-3,

nor purified IL-3 augmented the MLR (data not shown).

Figure 2 also shows that addition of antiserum abrogated not only the effect of exogenous cytokines but also the endogenous MLR of T cells to DC without Mφ or Mφ factors (Fig. 2). This was not caused by a nonspecific injurious effect of rabbit serum on mouse cells, because normal rabbit serum at the same concentrations did not affect the MLR (not shown). These results strongly suggest that GM-CSF play an essential role in the MLR.

Cells which respond to GM-CSF in the MLR. Before mixing cells in the MLR, DC, and responder T cells were pretreated separately with either the 25-kDa factor (Table IV) or rGM-CSF (Table V). When DC were pretreated with these factors, the subsequent MLR became significantly higher than that of untreated DC. The pretreatment of responder T cells with the 25-kDa factor was ineffective. As shown in Table V, the presence of anti-GM-CSF antiserum on the preculture of DC with 25-kDa factor or rGM-CSF abrogated the enhancing effect of these factors on the stimulatory activity of DC.

In contrast, anti-GM-CSF antiserum did not interfere with the enhanced activity of DC which had been pretreated with rGM-CSF or 25-kDa factor (Table VI). This implies that DC once triggered by GM-CSF have no need for the continual presence of GM-CSF to manifest the enhanced stimulatory activity.

Comparison of the effect of GM-CSF and IL-1 in Click's-NMS medium with that in RPMI 1640-FBS medium. The MLR in the above experiments was performed with Click's-NMS medium. The experiment was carried out to compare the effect of GM-CSF and IL-1 in Click's-NMS medium with that in RPMI 1640-FBS medium which is often used in MLR cultures (Table VII). The MLR stimulated by untreated DC was higher in RPMI-FBS medium than in Click's-NMS medium (19.5 vs. 10.3). In either culture medium, similar enhancing effect of rIL-1 was observed and was not blocked by the addition of anti-GM-CSF antiserum, irrespective of the use of IL-1 to pretreat the DC or to supplement the MLR. rGM-CSF augmented the MLR in Click's-NMS medium whether it was used to pretreat the DC or to supplement the MLR, but the enhancing effect of rGM-CSF was hardly observed in RPMI-FBS medium in either treatment of DC. As mentioned above, the MLR stimulated with untreated DC in Click's-NMS medium, but not in RPMI-FBS medium, was abrogated when an excess of anti-GM-CSF antiserum was present in the MLR culture continuously. The MLR performed in the presence of rGM-CSF was also

TABLE III
Synergism of IL-1 with the MLR-enhancing activity of Mφ supernatant

Dilution of IL-1	MLR ^a (cpm $\times 10^{-3}$) with Dilution of Mφ Supernatant ^b			LAF Activity ^c (cpm $\times 10^{-3}$) with Dilution of Mφ Supernatant ^b				
	None	1/2	1/4	1/8	None	1/2	1/4	1/8
None	1.6	6.0	1.6	0.8	1.9	2.1	1.3	1.5
1/8	6.3	53.2	17.5	6.2	42.9	32.6	29.9	39.4
1/16	6.7	44.3	12.8	7.5	35.7	34.6	25.0	30.9
1/32	6.2	37.7	11.5	4.2	21.8	26.5	20.7	ND
1/64	3.0	24.2	5.1	2.5	7.9	13.4	8.5	10.9
1/128	2.7	19.2	3.6	1.9	4.5	6.3	5.6	4.9

^a CD2F1 responder T cells (5×10^6 /culture) and B10.BR DC (1.5×10^6 /culture) were cultured in the presence or absence of Mφ supernatant and/or IL-1 partially purified by gel filtration. ³H-TDR incorporation of responder T cells alone was 737 in the absence of any factor, and 424 to 1189 in the presence of Mφ supernatant and/or IL-1.

^b Mφ supernatant was prepared from Mφ precultured with rIFN- γ (100 U/ml) for 4 days.

^c C3H/HeSk thymocytes (5×10^6 /culture) and PHA (1 μ g/ml) were used for LAF assay.

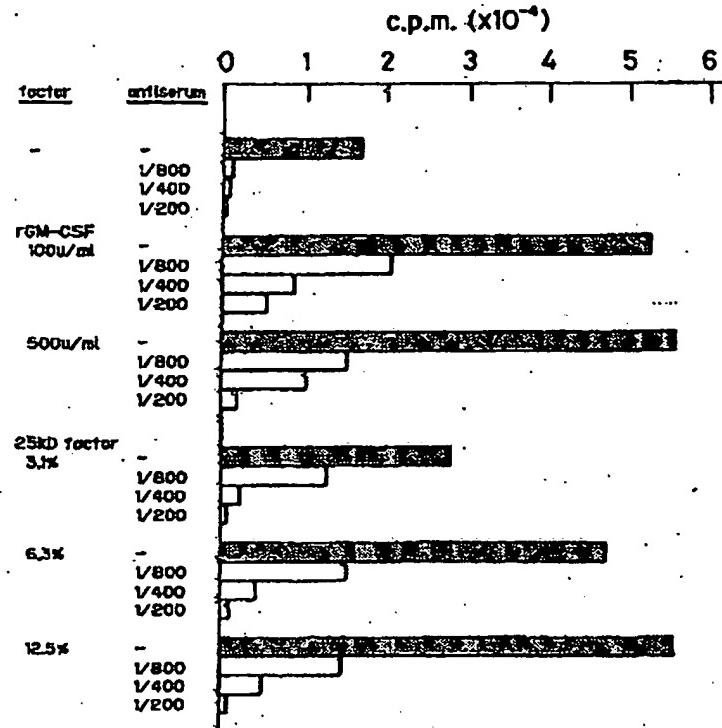


Figure 2. Effect of anti-rGM-CSF antiserum on the MLR in the presence or absence of rGM-CSF or 25-kDa factor. CD2F1 responder T cells (5×10^6) and C3H/HeSic DC (1.5×10^6) were cultured with the indicated factors and/or antiserum for 4 days. Partially purified 25-kDa factor was prepared by HPLC gel filtration. Normal rabbit serum at the concentration of 1/800 to 1/200 did not affect the MLR (data not shown).

TABLE IV
Effect of preculture of responder T cells or stimulator DC with the 25-kDa factor on the subsequent MLR*

Conc. of Factor ^b in Preculture of:		MLR (cpm $\times 10^{-4}$) with No. of DC ^c			
Responder (%)	DC (%)	3×10^3	10^3	3×10^3	10^3
12.5	-	1.7	8.0	29.5	92.2
	25.0	7.6	31.6	88.4	117.5
25.0	-	9.1	35.8	89.2	132.2
	12.5	1.8	8.4	30.1	98.6
12.5	25.0	1.7	8.4	28.8	88.7

* CD2F1 responder cells and C3H/HeSic DC were separately precultured overnight (16 h) in the presence or absence of the 25-kDa factor at the indicated concentrations (12.5 or 25%). FcR⁺ cells contaminating the precultured DC population, were removed by EA rosetting. The precultured cells were washed, and 3×10^3 of the responder cells and the indicated numbers of the DC were cultured in flat-bottomed 96-well plates. [³H]TdR incorporation of the responder cells alone was 106 to 114, whether precultured in the presence or absence of the 25-kDa factor.

^b HPLC fractions of 20 to 30 kDa were pooled and used as the 25-kDa factor.

more sensitive to the antiserum in Click's-NMS medium than in RPMI-FBS medium.

DISCUSSION

Although Mφ seem incapable of acting as direct stimulators of the primary allogeneic MLR, they enhance the MLR initiated by DC (1). Thus, a small number such as 0.2% DC plus appropriate doses of Mφ are comparable to 10 times as many as the number of DC in stimulating allogenic T lymphocytes. In this study, we present evidence that IL-1 and GM-CSF are Mφ products that enhance DC function.

We first noted that direct contact of DC plus T cells

TABLE V
Effect of anti-GM-CSF antiserum on the activation of DC by the preculture with rGM-CSF or 25-kDa factor*

Addition to Preculture of DC	MLR (cpm $\times 10^{-4}$) with No. of Precultured DC ^c	
	10^3	3×10^3
None	7.8	44.0
rGM-CSF	31.0	69.0
rGM-CSF + anti-GM-CSF antiserum	5.3	37.8
25-kDa factor	23.1	61.0
25-kDa factor + anti-GM-CSF antiserum	5.5	35.5

* DC were precultured overnight with or without rGM-CSF (200 U/ml) or the 25-kDa factor (25% v/v) in the presence or absence of anti-GM-CSF antiserum at 1/200 dilution. The stimulatory activity of precultured DC was assessed by the subsequent MLR for 4 days. [³H]TdR incorporation of culture of responder cells alone was 104.

TABLE VI
Effect of anti-GM-CSF antiserum on MLR to DC which had been precultured with rGM-CSF and the 25-kDa factor*

Addition to MLR Culture	MLR (cpm $\times 10^{-4}$) Stimulated by DC		
	Control	rGM-CSF	25-kDa factor
Name	11.5	34.5	30.8
NRS			
1/600	13.3	37.7	32.4
1/200	12.4	34.3	31.5
Anti-GM-CSF			
1/600	8.9	31.2	31.9
1/200	2.9	28.9	28.2

* One thousand of DC precultured overnight (16 h) with or without rGM-CSF (200 U/ml) or 25-kDa factor (25% v/v) were employed as stimulator cells. MLR proceeded in the presence or absence of indicated concentration of normal rabbit serum (NRS) or anti-GM-CSF antiserum.

MLR-ENHANCING FACTORS FROM MACROPHAGES

TABLE VII
Comparison of the effect of rGM-CSF, Mφ supernatant, rIL-1 and anti-GM-CSF antiserum on the MLR in Click's-0.5% NMS medium with that in RPMI 1640-10% FCS medium^a

DC Pretreated with:	Addition to Culture		MLR (cpm $\times 10^{-3}$) w/ban Cultured in	
	Factor	Anti-GM-CSF	Click's-0.5% NMS	RPMI 1640-10% FBS
No pretreatment	-	-	10.3	19.5
rGM-CSF	+	-	2.6	20.1
rGM-CSF	-	+	33.7	28.3
Mφ supernatant	+	-	7.1	24.2
Mφ supernatant	-	+	21.2	27.3
rIL-1	+	-	20.0	25.9
rIL-1	-	+	30.0	31.6
rGM-CSF	-	-	29.9	22.4
rIL-1	+	-	26.7	24.1
rIL-1	-	+	28.7	30.1
rIL-1	+	-	27.0	29.8

^a DC were pretreated with indicated factors overnight, and 1.5×10^6 of the DC were cultured with responder T cells (3×10^6) in the presence or absence of the factors either in Click's medium containing 0.5% NMS or RPMI 1640 supplemented with 10% FCS. Concentrations of factors: rGM-CSF, 1 ng/ml; rIL-1, 100 U/ml; Mφ supernatant, 25% v/v. Anti-GM-CSF antiserum was also added to some culture at the dilution of 1/400.

and Mφ was not necessary to detect enhancement of the MLR (Table I). In such double chamber system, both syngenic and allogeneic Mφ exhibit similar MLR-enhancing capacity. Aldehyde-fixed Mφ lose enhancing capacity even when co-cultured with DC and T cells (not shown).

The culture supernatant of freshly isolated Mφ exhibits both MLR-enhancing and LAF activities (Table II). Although this may appear to indicate that Mφ did not require the direct interaction with DC and/or T cells for secreting such factors, we suspect that the procedure used to prepare Mφ and their supernatant may serve as signals for factor production. The thioglycollate medium used to elicit PEC contains a variety of foreign Ag and LPS (11 ng/ml), and about 40 pg/ml LPS was detected by the *Limulus amoebocyte lysate assay* in Click's medium used for overnight culture. This amount of LPS may stimulate Mφ to secrete cytokines.

The MLR-enhancing activity of Mφ supernatant has been found to be due to two distinguishable categories of molecules. One is most probably IL-1, because it had a $M_r \sim 15$ kDa and has thymocyte mitogenesis activity (Fig. 1). The other is a 25-kDa molecule whose activity is neutralized by rabbit anti-mouse GM-CSF antiserum (Fig. 2), but not by normal rabbit serum (not shown). Mouse rGM-CSF also has a similar enhancing activity in the allogeneic MLR (Fig. 2). Furthermore, concentrated Mφ supernatant manifests a low but significant GM-CSF activity in a colony-forming assay for bone marrow cells in methylcellulose gel and this colony-forming activity is abrogated by anti-GM-CSF antiserum (not shown). These results strongly suggest that the 25-kDa factor is GM-CSF, though we cannot rule out the presence of an MLR-enhancing factor which is different from GM-CSF but reacts with the anti-GM-CSF antiserum used in this study. There is the possibility that other factors, such as IL-6 and TNF, produced by Mφ may enhance DC function.

Both factors seem to act primarily on DC, because preculturing DC, but not responder T cells, with either rGM-CSF, 25-kDa factor, or rIL-1 augments the MLR to a comparable level to that seen in the continuous presence of such factors in the MLR (Table IV, V, and VII). Addition of anti-GM-CSF antiserum to the preculture of DC with 25-kDa factor or GM-CSF abolished the effect of these factors on DC function (Table V). The augmentation

of DC function induced by GM-CSF seems to be related to the ability of DC to form clusters with T cells.

Our data suggest that GM-CSF and IL-1 affect DC function independently of each other. The enhancing effect of IL-1 on the MLR, that follows pretreatment of DC with or by continuous presence of IL-1 in the culture, is not abrogated by anti-GM-CSF antiserum (Table VII). Furthermore, IL-1 is effective in augmenting the MLR both in RPMI-FBS medium and in Click's-NMS medium, whereas GM-CSF is apparently ineffective in our RPMI-FBS medium (Table VII). Recently, Witmer-Pack et al. (17) reported that the addition of GM-CSF into the culture of freshly isolated epidermal LC resulted in the functional maturation of LC as stimulators or accessory cells, but not that GM-CSF had no effect on the stimulatory function of mature LC as of splenic DC.

The reason that the effects of GM-CSF and anti-GM-CSF antiserum are different in RPMI-FCS and in Click's-NMS medium is still unclear. FCS may contain some other factors than GM-CSF which activate DC, or bovine-GM-CSF activates murine DC but is not neutralized by anti-GM-CSF antiserum used in this study.

During the preparation of this manuscript, Morrissey et al. (18) reported that GM-CSF amplified the primary antibody response, suggesting that GM-CSF enhances accessory cell function of spleen adherent cells. An elevated expression of Ia Ag was shown (18), but we found no increase in DC Ia Ag after preculture with GM-CSF or 25-kDa factor (FACS analysis; not shown). These inconsistencies in the results may be due to the different accessory cell populations used in the experiments.

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Recombinant IL-12 Administration Induces Tumor Regression in Association with IFN- γ Production¹

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Recent evidence supports the critical and proximate role of IL-12 in regulating both T and NK cell function during inflammation. In these studies, we evaluated the *in vivo* antitumor activity of murine IL-12 in murine adenocarcinoma and sarcoma models using both systemic and peritumoral administration. Antitumor effects were consistently demonstrated both in models of microdisease, in which IL-12 treatment was initiated soon after tumor inoculation (1 to 5 days), and in animals bearing large established tumors (7 to 14 days). Treatment with IL-12 markedly prolonged survival and, in most cases, caused complete tumor regression. Significant reduction in pulmonary metastases after systemic treatment was observed when treatment was delayed for 10 days after tumor inoculation. Increases in serum IFN- γ , TNF- α , and nitrogen oxides were demonstrated, exceeding those observed with IL-2 treatment. Systemic administration of anti-IFN- γ Abs before IL-12 treatment nearly completely abrogated the antitumor effect in experiments using subcutaneous tumors or pulmonary metastases. Depletion of the individual T cell subsets CD4 and CD8 by systemic administration of mAbs diminished the effectiveness of IL-12 when administered in combination. An infiltrate composed primarily of CD8 $^{+}$ cells was demonstrated by using immunohistochemical analysis of tumors after IL-12 treatment. Minimal apparent toxicity was demonstrated at effective doses (0.1 to 1.0 μ g/day) of IL-12. These results indicate that IL-12 is an effective and minimally toxic antitumor agent in murine tumor models and leads to an immune-mediated rejection involving, at least in part, IFN- γ , CD4 $^{+}$, and CD8 $^{+}$ cells. Human clinical trials of IL-12 for the treatment of malignancy are supported by these studies. *The Journal of Immunology*, 1994, 153: 1697.

IL-12 was originally identified as a NK cell stimulatory factor (1) and a CTL maturation factor (2). The ability of this disulfide-linked heterodimer to regulate both T and NK cell function has been clearly established (3-5). Composed of two distinct subunits with molecular masses of 35 kDa and 40 kDa (6), the cDNAs encoding both subunits have recently been cloned and confirmed to produce biologically active IL-12 when expressed in COS-

cells (7). Secreted by macrophages and some B-cell lines, IL-12 induces NK and T cells to produce IFN- γ at high levels (4) and TNF- α to a lesser degree. In addition, IL-12 enhances NK lytic activity, alloreactive lymphocyte responses, and highly specific cytotoxic T cell responses (5). IL-12 not only facilitates a Th-1 cellular immune response (8), but also inhibits differentiation of Th-2 lymphocytes.

Because human IL-12 is species specific, murine studies were precluded until the recombinant protein became available (9). Administration of recombinant murine IL-12 (r-mIL-12)³ has *in vitro* effects similar to those observed with human IL-12, such as the ability to generate lymphokine-activated killer T cell activity and induce IFN- γ production from murine NK and T cells (10). In addition to its potent immune effects *in vitro*, recent experiments using systemic administration of IL-12 have demonstrated

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³ Abbreviations used in this paper: r-mIL-12, recombinant murine IL-12; GKO, gamma-IFN gene knockout; BID, twice daily; ELISA, enzyme-linked immunosorbent assay.

potent antitumor responses in models of local and metastatic disease. (11, 12) Furthermore, evaluation of these effects using nude mice and lymphoid subset depletions provide preliminary evidence for an immune-mediated phenomenon. In this report, antitumor effects of IL-12 are confirmed in a variety of tumor models. We demonstrate that both dose and timing of administration are important in determining the degree of antitumor response. The cellular infiltrate in these regressing tumors is characterized through immunohistochemical staining by a predominant increase in CD8⁺ lymphocytes. The antitumor response is sensitive both to sublethal irradiation and to CD4 combined with CD8 in vivo lymphocyte depletion with mAbs. Systemic treatment with IL-12 also induces high serum levels of IFN- γ , which are associated with tumor regression. Abs to IFN- γ , but not TNF- α , were able to reduce substantially the effectiveness of IL-12 therapy. Thus, IFN- γ may also be, in part, responsible for the antitumor properties of IL-12.

Materials and Methods

Mice

Female C57BL/6 mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Animals were age matched at 8 to 12 wk in all experiments and housed in groups of five. Routine periodic surveillance for mycoplasma and murine viruses was conducted in the Central Animal Facility, University of Pittsburgh.

Tumor cell lines

The MC-38 cell line was originally derived from a colon adenocarcinoma. The MCA-105 and MCA-207 methylcholanthrene-induced sarcomas were obtained from the National Cancer Institute (S. Rosenberg, Bethesda, MD) and maintained by serial transplantation in mice (13). Tumor lines were maintained as adherent cultures derived from harvesting fresh tumor specimens approximately 5 mm in diameter and digesting them with DNase, collagenase, and hyaluronidase (Sigma Chemical Co., St. Louis, MO) to obtain single-cell suspensions as previously described (14). Cell lines were maintained *in vitro* in RPMI 1640 medium supplemented with 10% heat-inactivated FCS, 2 mM glutamine, 100 μ g/ml streptomycin, 100 IU/ml penicillin, and 5.5 \times 10⁻⁵ M 2-ME (all from Life Technologies, Inc., Grand Island, NY). Cell lines were tested periodically by using a DNA hybridization probe (GeneProbe, Fisher Scientific, Pittsburgh, PA) and remained free from mycoplasma.

Cytokines and detection assays

The r-mIL-12 was kindly provided by Dr. B. Hubbard (Genetics Institute, Cambridge, MA). It was purified from the supernatants of Chinese hamster ovary cells transfected with the expression plasmids for p35 and p60. The specific activity was 5 to 7 \times 10⁶ U/mg as determined by the PHA blast proliferation assay previously described (15). SDS-PAGE analysis indicated that the IL-12 was \geq 95% pure, and endotoxin contamination was <5 units/mg IL-12, as assessed by the *Limulus* amoebocyte assay. For *in vivo* administration, IL-12 was diluted in 0.1% mouse albumin carrier protein. Highly purified recombinant human IL-2 with a specific activity of 18 \times 10⁶ International Units/mg was obtained from Am. Louis (Chiron, Emeryville, CA; 16, 17). IFN- γ and TNF- α serum measurements were obtained using ELISA kits purchased from Genzyme Corp. (IFN- γ -Cambridge, MA) and Life Technologies (TNF- α). Total serum nitrite was measured using HPLC determination of NO₂ + NO₃ levels.

Blocking Abs

Murine mAbs to IFN- γ and TNF- α were produced by R. Schreiber as previously described (18). Mice were injected i.p. with 1 ml of purified

Ab (0.25 mg/ml) before cytokine treatment. After Ab treatment, levels of each specific cytokine were undetectable by ELISA, even under appropriate inducing conditions. Anti-CD4 (ATCC clone GK1.5, rat IgG2b) and Anti-CD8 (ATCC clone 2.43, rat IgG2b) mAbs were prepared as previously described (11). These Abs were administered as i.p. injections (1 mg) 18 h before cytokine treatment. Standard two-color flow cytometry was performed using FACScan (Becton Dickinson, Mountain View, CA), verifying depletion of specific cell subsets in the spleen after the administration of blocking Abs.

Immunohistochemistry

Tumor specimens were snap frozen and harvested 5 to 7 days after IL-12 or diluent treatment. Cryostat cut sections were fixed in cold acetone, hydrated in PBS, and incubated in protein blocking solution (Lipshaw Immunon, Pittsburgh, PA) for 8 min. They were then incubated overnight at 4°C with rat mAbs directed against mouse CD4, CD8, NK cells, or macrophages (MOMA-2, Serotec Ltd., Oxon, UK). Isotype-matched rat IgG was used as a negative control. A biotinylated mouse primary Ab against I-A^b was also employed. In this case, an irrelevant biotinylated mouse mAb (Anti-BRDU, Caltag Laboratories, San Francisco, CA) was used as a negative control. Positive tissue controls consisted of frozen sections of spleens from B10 and BALB/c mice. After buffer washes, endogenous peroxidase activity was quenched with 0.6% H₂O₂ in methanol. Species-absorbed biotinylated mouse anti-rat F(ab')₂ (Jackson ImmunoResearch Labs, Inc., West Grove, PA) was applied to sections incubated with unlabeled primary Abs, followed by streptavidin-peroxidase (Boehringer Mannheim, Indianapolis, IN) and 3'-amino-9-ethylcarbazole (Biomedica Corp., Foster City, CA). In the case of anti-I-A^b and its negative control, the biotinylated secondary Ab was eliminated. Sections were counterstained with hematoxylin and mounted in CrystalMount (Biomedica Corporation).

Animal experiments

Groups of 5 to 10 mice were ear tagged and randomized before inoculation with the syngeneic tumors MC-38 adenocarcinoma, and MCA-105 and MCA-207 methylcholanthrene-induced sarcoma (1 to 5 \times 10³ cells) s.c. Treatment with peritumoral or i.p. daily injection of IL-12 (0.1 to 1.0 μ g/day) was initiated on various days postinoculation. Serial microcaliper measurements of perpendicular tumor diameters were obtained in a blinded fashion, were used to calculate tumor volume (mm^3 = longest diameter \times shortest diameter²), and were subsequently verified in some experiments by weight. In experiments involving tumors inoculated on both flanks, one side received daily IL-12 injection while both the ipsilateral and contralateral sides were monitored for tumor growth. Mouse serum was obtained during the experiments through tail vein phlebotomy. Depleting Ab was administered by tail vein or i.p. injection 18 h before cytokine treatment as indicated in figure legends.

Pulmonary metastases model

Pulmonary metastases were seeded by direct tail vein injection of 1.5 to 5.0 \times 10⁵ cells suspended in 0.5 ml serum-free BSS. Blocking Abs were administered i.v. 18 h before initiation of cytokine treatment. Ear tags identified mice for randomization into treatment groups receiving daily i.p. injections of diluent vs cytokine from day 7 to day 14, alone or in combination with IL-2. Animals were killed on day 21, and lungs insufflated with 15% India ink and bleached in Fekete's solution (19) to enumerate pulmonary metastases.

Statistical analysis

Calculated tumor sizes were compared for each group and compared with other groups using a standard nonparametric Wilcoxon rank test. Differences were considered significant when $p < 0.05$.

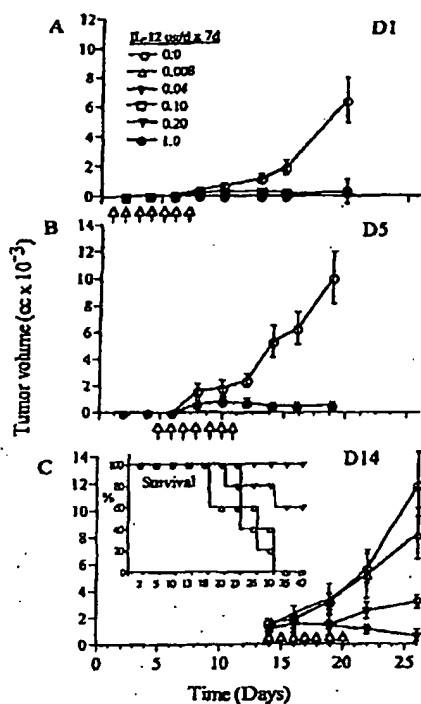


FIGURE 1. IL-12 significantly delays tumor growth in mice bearing s.c. tumors and significantly prolongs survival. In this series of studies, the effect of peritumoral administration of IL-12 on MCA-105 tumor growth is demonstrated in animals treated on day 1 postinoculation (**A**, D1), on day 5 (**B**, D5), and on day 14 after tumor inoculation (**C**, D14). The period of daily IL-12 injections corresponds to the open arrows located on the abscissa. Tumor volume is presented for groups of 5 mice using the product of microcaliper-determined perpendicular diameters obtained in a blinded fashion from mice randomized by ear tag number. Error bars in each graph represent mean \pm SEM. In Figure 1A, all mice treated at the highest dose of IL-12 (1.0 μ g/day) failed to develop palpable tumor, whereas two of five animals treated at a lower dose (0.1 μ g/day) developed palpable tumors that subsequently regressed. Treatment initiated on day 5 (Fig. 1B) was associated with initial tumor growth in all animals, and subsequent tumor regression. A day-14 tumor model is presented in Figure 1C, in which animals bearing a substantial tumor burden (approximately 1 cm) are treated at doses of IL-12 ranging from 0.2 μ g to 0.008 μ g daily from day 14 through day 21. At the highest doses tested in this experiment (0.2 μ g/day) and in subsequent experiments (1.0 μ g/day), virtually all of these larger tumors underwent regression. A dose-response relationship was observed in this experiment. A survival curve is presented in the inset demonstrating a prolonged survival to day 40 in animals treated at higher doses. No survival data is presented for Figures 1, **A** and **B** as treatment was associated with prolonged survival in all cases until animals were killed or rechallenged with parental tumor. Experiments were repeated a minimum of two times for each

Results

IL-12 administration causes regression of s.c. MC-38 and MCA-105 sarcoma and prolongs survival in established tumors

In a series of studies using peritumoral administration of IL-12 after s.c. inoculation of tumor, antitumor effects were demonstrated in each of 18 experiments. Figure 1, **A** through **C** demonstrates the effect of daily peritumoral injection of r-mIL-12 on the progression of MCA-105 sarcoma. When treatment was initiated early (day 1, Fig. 1A), animals failed to develop tumor at the highest dose administered (1.0 μ g/day). At lower doses, initial tumor emergence was frequently seen as a palpable lesion that subsequently regressed. Treatment beginning on day 5 was associated with development of a palpable tumor; however, tumor growth was significantly delayed and regression was demonstrated in the majority of cases (Fig. 1B). Animals bearing large established tumors (approximately 1 cm) were randomized and treated beginning on day 14. In these animals, virtually all of these larger tumors underwent regression at the highest doses of IL-12. A dose response relationship was demonstrated from 0.2 μ g to 0.008 μ g daily (Fig. 1C). The inset demonstrates the corresponding increase in survival of the treated animals. Survival curves are not presented for Figures 1, **A** and **B** as treatment was uniformly associated with survival, whereas diluent-treated controls all succumbed to tumor progression. Experiments were repeated a minimum of two times for each condition, demonstrating the reproducibility of these antitumor effects. Additional experiments demonstrated systemic effects of IL-12 administration; in animals inoculated with bilateral tumors, regression occurred not only in the tumors directly injected with IL-12, but also in the contralateral tumors (Fig. 2). In studies comparing the systemic (i.p.) route of administration vs the peritumoral route, antitumor effects were equivalent (data not shown). Table I lists experiments with several tumor models that demonstrate partial to complete antitumor responses in animals treated with IL-12. In 7-day established tumors, complete tumor regression was documented in most cases. Significant tumor regression was also demonstrated in day-14 models at the highest doses. The subsequent ability to reject 1×10^6 cells intradermally was noted in the majority of animals tested after complete tumor regression.

Systemic IL-12 administration significantly reduces MC-38 pulmonary metastases in advanced 10-day treatment models

To determine whether the antitumor effect of IL-12 could be extended to a model of metastatic disease, experimental

condition with similar antitumor effects seen. In some cases, animals were killed for immunohistochemical analysis of growing or regressing tumors.

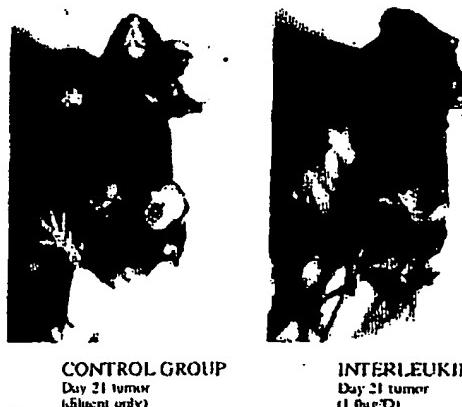
TREATMENT WITH INTERLEUKIN-12 SUPPRESSES *IN VIVO* TUMOR GROWTH

FIGURE 2. Treatment with IL-12 suppresses *in vivo* tumor growth. Groups of 10 animals were injected on day 0 with bilateral tumors located on each flank. Animals were injected peritumorally in the right flank with 1 µg IL-12 or diluent daily from day 5 through 12 after tumor inoculation (MCA-105 sarcoma, 1×10^6 cell/inoculum). Regression was observed both in the tumor treated directly and a sentinel tumor on the contralateral (left) flank. Control animals showed progressive bilateral growth of tumors. Animals above were photographed on day 21.

pulmonary metastases were established by injecting animals with 1 to 5×10^6 MC-38 adenocarcinoma cells on day 0. Treatment of experimental pulmonary micrometastases with systemic IL-12 was initiated beginning on day 10 postinoculation. In a series of day-10 models, pulmonary metastases were significantly reduced after systemic treatment with IL-12 at a dose of 1 µg/day (Fig. 3, group

2 vs 1, $p = 0.05$). Systemic IL-2 caused effective reduction of pulmonary metastases in this model when administered at high dose (group 4 vs 1, $p < 0.05$) although associated with toxic systemic effects. The combination of these individual cytokines at low doses was ineffective under these conditions. Repeat experiments confirmed a 72% ± 14% reduction in pulmonary metastases with IL-12 treatment alone ($p < 0.05$, $n = 5$).

In vivo IL-12 administration is associated with elevated serum IFN-γ and nitric oxide levels

Serum IFN-γ levels became detectable as early as 12 h after a single administration of i.p. IL-12. When determined after chronic daily IL-12 administration, IFN-γ levels remained significantly and dramatically elevated during therapy ($p < 0.001$) and declined after discontinuation (Fig. 4). TNF-α was detectable in treated animals only at low levels after chronic IL-12 administration. Similarly, IL-12 treatment led to elevated serum total nitrite and nitrate levels approximating those observed in sepsis or severe inflammation. Nitrate levels remained elevated for 48 h after discontinuation. Similar profiles of serum cytokine elevation were observed in tumor-bearing animals.

Immunohistochemical evaluation of regressing tumors is characterized by a predominantly CD8⁺ cellular infiltrate

After staining of MCA-207 sarcoma with mAbs to T cell subsets, NK, macrophage, and class II cell surface markers, a specific cellular CD4 and CD8 infiltrate was identified. Overall, the CD8/CD4 ratio was 20:1 to 40:1, with the highest concentration of CD8⁺ cells in the periphery of the tumor. The infiltrate was characterized by perivascular chronic inflammation of predominantly CD8⁺ lymphocytes (Fig. 5). Tumors graded in a blinded fashion demonstrated significant differences in number of CD8⁺ infiltrating cells with no significant difference in the degree of

Table I. Experiments with several tumor models demonstrating partial to complete antitumor responses in animals treated with IL-12

Exp. No.	Tumor	Inoculum	Day Rx	Dose (µg)	N	PR	CR	Rechal	Immune
1	MCA-105	105	3-10	0.1-1.0	15	12	3	2	1
2	MCA-105	3×10^6	3-10	0.1-1.0	15	10	5	3	2
3	MCA-105	106	5-12	1.0	10	10	0	ND ^a	ND
4	MCA-105	105	14-21	0.008-2.0	15	4	1	ND	ND
5	MC-38	105	1-8	0.1-1.0	10	4	6	3	3
6	MC-38	105	5-12	1.0	15	6	8	ND	ND
7	MC-38	105	7-14	1.0	10	7	3	3	3
8	MC-38	105	7-14	1.0	10	3	7	ND	ND
9	MC-38	105	14-21	0.008-2.0	15	4	1	ND	ND
10	MCA-207	3×10^5	5-12	0.1-1.0	15	12	3	ND	ND
11	MCA-207	3×10^5	7-14	0.1-1.0	10	6	4	ND	ND
12	MCA-207	3×10^5	7-14	0.1-1.0	25	10	15	15	14
13	MCA-207	3×10^5	7-14	0.1-1.0	20	4	16	16	14
14	MCA-207	3×10^5	7-14	0.1-1.0	15	3	12	ND	ND

^a ND, not determined.

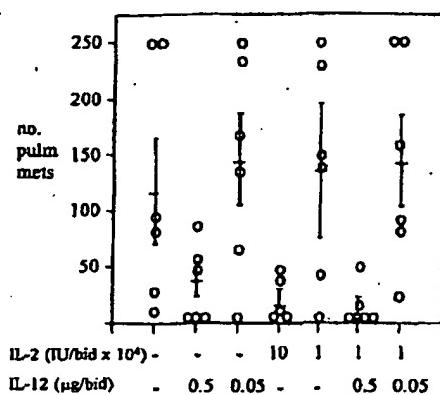


FIGURE 3. Reduction in MC-38 pulmonary metastases after IL-12 treatment alone or in combination with low-dose IL-12. Pulmonary metastases are assayed on day 24 after 7 days of cytokine therapy initiated on day 10. Cytokine injections in this experiment were administered i.p. at doses presented along the abscissa and were given twice daily to allow randomization with and comparison to IL-2 therapy. Mean number of metastases per group \pm SEM are presented as horizontal dashes with error bars. Each animal in this experiment is represented by an open circle. This graph is representative of three experiments with the groups listed from left to right as follows: diluent control; IL-12 0.5 μ g BID; IL-12 0.05 μ g BID; IL-2 100K IU BID; IL-2 10K IU BID; combination therapy with IL-12 (0.5 μ g) and IL-2 (10K IU) BID; combination therapy with IL-12 (0.05 μ g) and IL-2 (10K IU) BID. High dose IL-12 treatment (0.5 μ g/mouse bid) alone significantly reduced the number of metastases ($p < 0.05$) whereas low dose had no effect. Systemic IL-2 given in this fashion also substantially reduces the number of pulmonary metastases at high dose but not low dose. Although the combination of cytokines was effective at the highest dose, the lower dose remained ineffective in combination.

NK or macrophage infiltrate. Interestingly, this lymphocytic infiltrate differs from the characteristic dense infiltration of macrophages surrounding regressing tumors after IL-12 treatment delivered by genetically engineered fibroblasts in our previous report (20).

Depletion of both CD4 and CD8 subsets reduce the IL-12-induced antitumor response to MCA-207

Because immunohistochemical data demonstrated a significant T cell infiltrate, the effect of depleting T cell subsets on tumor growth was examined. In an initial series of experiments, anti-CD4 and anti-CD8 Abs were administered before IL-12 treatment of MC-38 and MCA 207 tumors. Phenotypic analysis of peripheral blood and splenocyte populations, using standard two-color flow cytometry after Ab administration, confirmed $>95\%$ depletion of targeted lymphocytes. Although the control populations of T cell subsets were small (6.3–10.9% for CD8 $^{+}$, and approx-

imately double that for CD4 $^{+}$), spleens from depleted animals yielded T cell subset values below the limits of detection (0.12–0.23%), confirming $>95\%$ depletion. Selective depletion of the individual T cell subsets, CD4 and CD8, had no effect on the antitumor response induced by IL-12 in each of three separate experiments. However, when these Abs were administered concomitantly, near complete loss of antitumor activity was seen (Fig. 6). Similar results were confirmed in repeat experiments. Immunosuppression associated with sublethal total body irradiation (700 rad) increased the rate of tumor growth compared with control, and substantially abrogated the effectiveness of IL-12 treatment (Fig. 7).

Antibodies against IFN- γ but not TNF- α block ability of IL-12 to cause regression of s.c. tumors and partially reduce effectiveness of IL-12 against MC-38 pulmonary metastases

Neutralizing effects of mAbs against IFN- γ or TNF- α have been previously characterized (18). In our experiments, differential effects of blocking Abs on the antitumor effects of IL-12 were noted (Fig. 8). Significant inhibition of these effects in s.c. models was observed with administration of anti-IFN but not anti-TNF Abs ($p < 0.01$, IL-12 + anti-IFN vs IL-12) from days 29 through 35. Administration of these Abs to tumor-bearing animals in the absence of IL-12 therapy consistently accelerated tumor growth compared with diluent controls, as shown. In similar Ab blocking studies using the 10-day MC-38 pulmonary metastases model (Fig. 9), effectiveness of systemic IL-12 was demonstrated in groups 2, 3 and 4 vs 1 ($p = 0.02$, 0.04, and 0.02, respectively) and approached significance ($p = 0.07$) for group 5, in which the combination of anti-IFN and anti-TNF was used. In this model, anti-IFN and anti-TNF Abs alone or in combination demonstrated a trend toward reduction of the effectiveness of IL-12 ($p = 0.19$, 2 vs 3).

Discussion

In these studies, we demonstrate potent antitumor effects of IL-12 in a variety of tumor models, which in aggregate suggest an immune-mediated mechanism. IL-12 treatment was effective in animals bearing s.c. tumors or metastatic disease, consistent with previous observations (11, 12). The antitumor effect was seen with both systemic and peritumoral injection, causing regression even in tumor models as late as day 14. We further demonstrate that some components of the specific immune response are required for the antitumor effects. IL-12 administration was associated with dramatically increased serum levels of IFN- γ , consistent with recent reports of the in vivo immunologic effects of IL-12 (21), as well as increased TNF- α and nitric oxide levels. Evidence suggests that IFN- γ may be a proximate mediator of these effects. Furthermore, T cells are clearly implicated in these antitumor effects, demonstrated

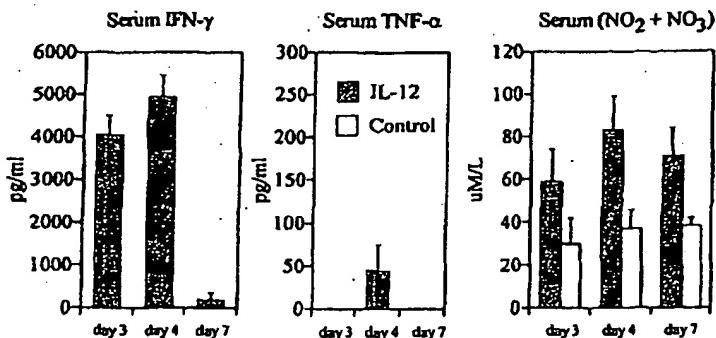


FIGURE 4. Induction of IFN- γ , TNF- α , and nitric oxide following in vivo administration of IL-12. Presented in this series of figures are cytokine and total nitrogen oxides (serum nitrate + nitrite) levels on day 3, 4, and 7 from nontumor bearing animals, $n = 5$ per group. In vivo administration of IL-12 is associated with significantly increased levels of a variety of cytokines known to possess both antitumor and antimicrobial effects. Among these are IFN- γ , TNF- α and nitric oxide, as measured by its stable end products nitrite and nitrate. These animals were treated for 5 consecutive days with i.p. IL-12 1.0 μ g/day. Serum IFN- γ levels are detectable in high levels after 3 to 4 days of IL-12 administration (left panel). Elevated levels become detectable as early as 6 h after administration (data not shown). Two days after discontinuation, levels fall below the limit of detection. Control animals exhibit no detectable amounts of IFN- γ . Serum TNF- α (center panel) becomes detectable only at low levels after chronic administration in treated animals (day 3 in this experiment) and is undetectable in vehicle controls. Nitric oxide end products (right panel) become elevated as early as day 3 and remain elevated for 48 h after discontinuation of IL-12.

by phenotypic and functional analysis. Peritumoral CD8 $^{+}$ infiltration was present in vivo and systemic blocking Abs abrogated these effects.

In our experiments, specific depletion of both lymphocyte subsets, CD4 and CD8, was required to abrogate the antitumor effects of IL-12. This is in contrast to the findings of others that show CD8 $^{+}$ depletion alone caused a partial reduction in antitumor activity in a murine renal cell model (11). Despite confirming the extent of subset depletion by phenotype analysis, it is possible that the splenocyte compartment did not reflect the adequacy of functional depletion. Our results, which demonstrate substantially reduced antitumor effects after simultaneous depletion of both T cell subsets, suggest that CD4-provided T cell help may be important in these effects. This finding supports a previous observation that the efficacy of IL-12 is reduced in nude mice but not entirely eliminated (11). Immunosuppression induced by high-dose total body irradiation inhibits these effects as well.

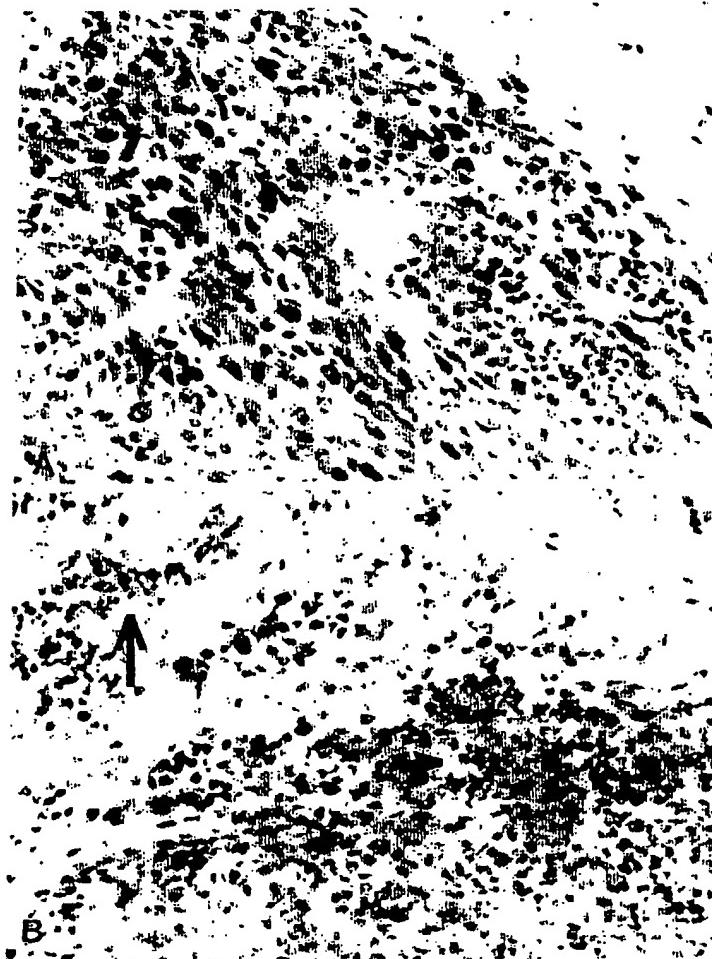
Our studies demonstrate that anti-IFN- γ but not anti-TNF- α Abs cause a loss of antitumor effects, suggesting that IFN- γ may also, in part, be responsible for the antitumor effects of IL-12. Although previous studies have shown that administration of IFN- γ alone may cause tumor regression and antiproliferative effects, its antitumor effects are much less pronounced than those reported here with IL-12. Additionally, in tumor models used in these studies, IFN- γ has no significant effect on cultured tumor cells (data not shown). Thus, although it may be possible to attribute the antitumor effects in these models to the dramatic elevations in systemic IFN- γ , it is unlikely to be solely the direct mediator of these effects. Significant ab-

rogation of IL-12 activity by using neutralizing anti-IFN- γ Abs suggests that IFN- γ may be a more proximate mediator while a variety of end-effector mechanisms may be involved.

The mechanism whereby IL-12 mediates its antitumor effects would be anticipated to involve multiple factors, including the activation and expansion of cellular immune circuits as distinguished from humoral. Cellular responses are promoted through specific augmentation of the lytic activity and proliferation of CTL clones in vitro (5) and the ability to induce preferentially Th-1-type cells from naive precursors (8). Recently, the notion of IL-4 and IL-12 reciprocally modulating the development of Th0 cells has been presented (22), as both Th0 and Th2 cells appear to make IL-4. Because IL-12 is both a growth factor for activated T and specific CTL clones (5) and also serves to promote the generation of Ag-specific CTL in vitro and in vivo, one might expect the predominant effectors to be T cells. This is suggested by the significant CD8 $^{+}$ infiltration in all tumors derived from IL-12-treated animals. Additional evidence is suggested by depletion of both CD4 and CD8 subsets abrogating antitumor activity. Although depletion of NK cells with asialo-GM1 has little effect on the observed antitumor response, IL-12 may potentially enhance lysis of tumor cells mediated by LAK/NK, Ab-dependent cell-mediated cytotoxicity (23), as well as activated monocytes.

Why should IL-12, a cytokine promoting cellular immune responses and IFN- γ induction, have more potent effects than IFN- γ alone, if the antitumor effects are caused solely by IFN- γ production? First, this discrepancy may be explained by the difficulty of achieving adequate

FIGURE 5. Immunohistochemical evaluation of regressing s.c. tumors reveals a specific CD8 and CD4 cellular infiltrate. Tumors were harvested from 5 to 7 days after initiation of cytokine treatment. Fresh frozen specimens were evaluated using a panel of murine specific Abs to CD4, CD8, monocytes, NK cells, and MHC class II determinants. These tumors were evaluated by an independent pathologist blinded to the treatment given. Specific cellular infiltrate in IL-12-treated tumors consisted of a predominantly CD8⁺ infiltrate surrounding the tumor and a variable number of positive staining cells penetrating the tumor. Increased numbers of CD4⁺ cells were also present in treated tumors; however, the CD8:CD4 ratio ranged from 20:1 to 40:1. In Figure 5A, a representative diluent-injected control animal shows minimal peritumoral inflammation. At medium power magnification (100 \times), the anti-CD8 immunohistochemical stain demonstrated a significant peritumoral infiltrate (Fig. 5B, right arrow).



local production of IFN- γ with systemic administration. Factors limiting the effectiveness of IFN- γ in vivo include its short half-life (24), requiring frequent dosing, and systemic toxicity which may prevent optimal *in situ* IFN- γ production necessary for tumor eradication. Second, IFN- γ may exert its effects by augmenting the immune response in several ways: increasing Ag presenting capability by up-regulating both MHC class I and class II Ags, (25, 26), by activating macrophages (27), and by enhancing growth and differentiation of CTL (28–30). These effects may be enhanced by the differential kinetics of IFN- γ production when it is induced by the appropriately matured cells after chronic IL-12 administration and is active *in situ* over a longer time frame, rather than when directly administered. A variety of studies using tumors genetically engineered to produce IFN- γ have shown local IFN- γ secretion may be critically important in inducing an antitu-

mor response (31). In models of gene transfection into neuroblastoma (32), fibrosarcoma (33), and Lewis lung carcinoma (34), these tumors display reduced tumorigenicity and facilitate the induction of specific antitumor immunity, whereas anti-IFN- γ Abs suppress these antitumor effects. These effects could potentially be explained by low-dose cytokine that remains *in situ* over a longer time interval. Previous studies have demonstrated that neutralizing Abs to IFN- γ may also abrogate the antitumor effects of peritumoral IL-2 injections (35), ablate the antitumor effects of TIL (36), inhibit the ability of tumor draining lymph nodes to reduce pulmonary metastases (37), and, when given alone, may enhance tumorigenicity. (38) Thus, the effectiveness of IL-12 therapy may involve not only the local production of IFN- γ , but also additional cytokine cascades which affect chemotactic activity or cellular activation critically important in antitumor responses.

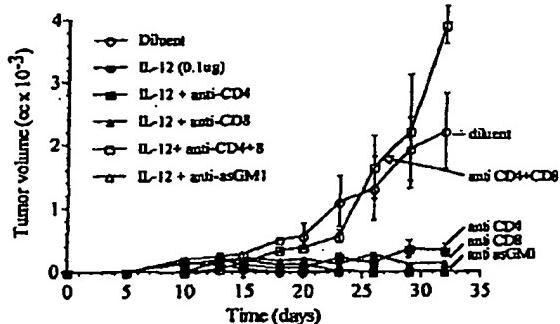


FIGURE 6. Selective lymphocyte depletion of both cellular subsets CD4 and CD8 abrogates the activity of IL-12 in the treatment of MCA-207 tumors. Anti-CD4 and anti-CD8 Abs were administered i.p. on day 6, 18 h before the initiation of IL-12 treatment on day 7. Abs were administered at a dose of 1 mg each or in combination. Depletion of cellular subsets >95% was confirmed (see results section) in selected animals using standard flow cytometry techniques employing splenocyte mononuclear cells. While pretreatment with either Ab alone did not reduce effectiveness of IL-12, the combination of the two Abs administered together blocked IL-12 antitumor effects. Depletion using anti-asGM1 had no significant effect on the antitumor activity of IL-12.

IL-12 may alter selected properties of tumor cells that contribute to their ability to evade an immune response. By increasing the cytolytic activity of T and NK cells, altering the production of other cytokines, causing lymphocyte proliferation and emigration into tissues, or biasing the relative number of CD4 and CD8 cells present, IL-12 may enhance local immune reactivity. Several cytokines, such as IL-2 and IL-12, facilitate the generation of human CTL in vitro. IL-12 plays a central role regulating lymphocyte function and number by acting as an IL-2 independent growth factor for CD4⁺ and CD8⁺ mitogen activated T lymphocytes (10); and enhancing the proliferative response of appropriately activated T cells to IL-2.

IL-12 also induces the production of nitric oxide in vivo, an effector molecule important in the host antiparasite relationship which may also be critical in antitumor cellular cytotoxic mechanisms (39). Studies examining the relative importance of nitric oxide in the IL-2 induced antitumor response vs its toxic side effects demonstrate that aminoguanidine, an inhibitor of nitric oxide production, decreases the toxic effects of IL-2 without affecting antitumor activity (Lotze, unpublished observations). IL-12 induces nitric oxide serum elevations comparable to levels seen in sepsis, equivalent to or greater than after treatment with IL-2. The apparent toxicity of IL-12 is far less, indicating that nitric oxide production may not be the principal mediator of toxicity. Although mice with disrupted gamma IFN genes (GKO) (40) do not produce IFN- γ in response

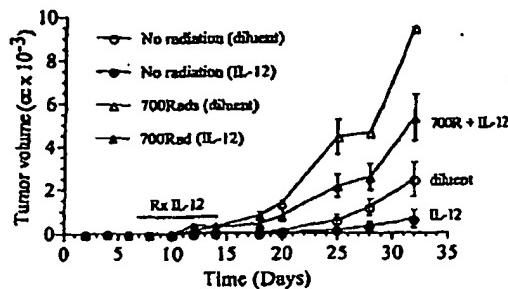


FIGURE 7. Radiation sensitivity of IL-12 therapy. Animals were irradiated with a sublethal dose (700 rad) on day 6 after tumor inoculation of 1×10^3 MC38 tumor cells, one day before treatment with systemic IL-12 (1 μ g/day as indicated by the horizontal bar on the abscissa). Irradiation alone increased the rate of tumor growth compared with control. Three of five animals died in the irradiated untreated group accounting for the low standard error presented. After irradiation and subsequent treatment with IL-12, all animals survived until day 32, but were killed with large, ulcerated tumors. When analyzed until day 25, IL-12 did not significantly affect tumor progression in irradiated animals. IL-12 treatment in nonirradiated controls significantly diminished tumor growth ($p \leq 0.05$, days 28, 32). These results were confirmed in a repeat experiment.

to IL-12, they do exhibit other systemic effects observed after IL-12 administration, such as a significant increase in splenic weight indicating that at least some effects of IL-12 are independent of IFN- γ production (Lotze, unpublished observation). Whether the antitumor response is impaired in these animals demands investigation; however, the developmental and immunologic compensatory mechanisms in these mice will add considerable complexity to the design and interpretation of such studies.

Previously, IL-2 has been considered the prototypic cytokine for in vivo cytokine immunotherapy. Although it causes regression of established tumors in a minority of patients (41) and in experimental animals, (42) severe toxicities are associated with IL-2 administration, largely linked to high levels of IL-2-induced serum TNF- α . (43) Large quantities of TNF- α production in vivo have been demonstrated to result in cachexia, capillary leak syndrome, and hemorrhagic necrosis. In contrast, IL-12-associated toxicity in vivo appears minimal. This may, in part, result from the fact that serum levels of TNF- α induced by IL-12 are far less than those induced by IL-2. Consequently, IL-12 is an attractive candidate for clinical trials by virtue of these in vivo studies demonstrating antitumor effects associated with minimal toxicity. In vitro data, suggesting potential synergistic effects with IL-2, may also be a strategy for optimizing immunotherapeutic approaches using lower concentrations of individual cytokines. As the

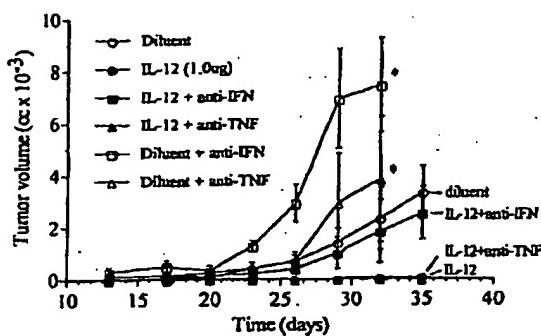


FIGURE 8. Cytokine depletion alters IL-12 effect on subcutaneous tumors. Anti-IFN- γ and anti-TNF- α Abs were administered on day 6 following inoculation of 3×10^5 MC-38 sarcoma cells, 18 h before the initiation of IL-12 treatment on day 7, and carried through day 14 (0.5 μ g/d). IL-12 treatment alone or in combination with previous administration of anti-TNF Abs demonstrated consistent antitumor effects (closed circles, closed triangle, respectively). When anti-IFN- γ Abs were administered before IL-12 treatment, antitumor effects were significantly reduced (closed squares) with tumor progression not significantly different from the control group. Abs to IFN- γ or TNF- α alone significantly accelerated tumor growth compared with vehicle alone. Animals were killed where indicated (*) because of progressive growth of tumor.

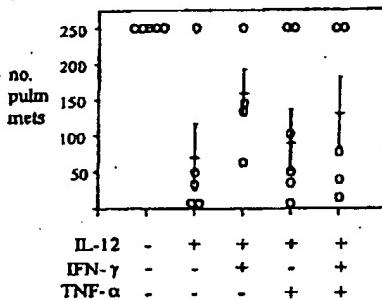


FIGURE 9. Depletion of IFN- γ in combination with TNF- α partially decreases the antitumor effect of IL-12 on pulmonary metastases. Animals received systemic treatment with IL-12 beginning in day 10 after tail vein injection of tumor. Pulmonary metastases were assayed on day 24 after 7 days of cytokine therapy initiated on day 10. Eighteen hours before treatment, animals received a single dose of 1 mg of depleting Ab to IFN- γ or TNF- α as noted in the figure. Groups 2 through 4 (left to right) received IL-12 treatment alone or in combination with blocking Abs. A significant decrease in mean number of metastases was demonstrated for groups 2, 3 and 4 ($p = 0.02$, 0.04 and 0.02 , respectively) vs control (group 1). In animals receiving a combination of the two Abs, the effectiveness of IL-12 was reduced ($p = 0.07$ for group 5 vs 1). In animals receiving depleting Ab, the specific cytokine was not detectable in serum. While the effectiveness of IL-12 in reducing pulmonary metastases was partially reversed with anti-IFN- γ Abs, this did not reach statistical significance ($p = 0.19$, 2 vs 3).

mechanism of IL-12 antitumor activity is more clearly defined, appropriate combinations of cytokines can be investigated systematically. Recent investigation by our group using IL-12 transfected fibroblasts as a means to locally deliver IL-12 (20) provided additional results supporting the clinical use of IL-12 gene therapy. These and our present results demonstrate potent antitumor effects of IL-12 in murine models and serve as a rationale for both continued investigation into the basic immunobiology of IL-12 and as a basis for initiating phase I human trials of systemic IL-12 administration.

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Recombinant interleukin 2 therapy in severe combined immunodeficiency disease

(T-cell growth factor/primary immunodeficiency disease/immunotherapy)

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ABSTRACT Severe combined immunodeficiency disease (SCID) is a congenital disorder of severe B- and T-lymphocyte dysfunction in which several pathogenic mechanisms have been identified. The present study describes a female child with SCID who had a primary defect in the ability of T cells to secrete interleukin 2 (IL-2). B- and T-cell numbers were normal, but their functions were severely deficient. Mitogen and antigen-driven lymphoproliferative responses were diminished but were correctable *in vitro* with recombinant IL-2 (rIL-2). The patient's phytohemagglutinin-stimulated lymphocytes expressed IL-2 receptors normally. Despite the presence of the gene for IL-2, the patient's cells were grossly deficient in messenger RNA for IL-2 and endogenous IL-2 production. Pokeweed mitogen-driven B-cell differentiation was decreased and was not corrected by the addition of normal T cells to the B cells. Two attempts at immune reconstitution by haploidical bone marrow transplantation failed. Therapy with rIL-2 (30,000 units/kg, given daily i.v.) resulted in marked clinical improvement as well as improved T-cell functions. The child, now 3 yr old, has been on rIL-2 therapy for 2 yr and receives rIL-2 (30,000 units/kg) three times weekly at home. This case study points to a new direction in the treatment of such disorders with rIL-2.

Severe combined immunodeficiency disease (SCID) is a congenital disorder of immune dysfunction involving the B- and T-cell systems. The condition is uniformly fatal if untreated. The pathogenic mechanisms involved in the evolution of the SCID syndromes are varied, the classical form being that of a "stem cell" defect (1). A multitude of other possible defects, usually linked to arrested T-cell differentiation (2–5), have been described to explain the pathogenesis of this disease. In this communication, we describe a female child with SCID whose disease could be attributed to a deficiency in synthesis and secretion of interleukin 2 (IL-2).

The initiation of T-cell proliferation involves a cascade of biochemical events culminating in induction of IL-2 receptor (IL-2R) expression and endogenous IL-2 production (6–11). The T-lymphocyte antigen-receptor complex (CD3Ti) recognizes processed antigen in association with major histocompatibility complex II determinants on the antigen-presenting cell (12). This recognition is transduced into intracellular biochemical events that result in a biologic response. The inability to synthesize adequate amounts of IL-2 has been reported to cause several types of T-cell dysfunction, including SCID (13–20). Successful treatment of SCID has thus far been possible only with bone marrow

transplantation (BMT). Thus, the trial reported here supports the view that SCID represents several different diseases and demonstrates the potential usefulness of recombinant IL-2 (rIL-2) in the treatment of primary defects of T-cell function secondary to a deficiency of production of IL-2.

MATERIALS AND METHODS

Isolation of Lymphocytes. Mononuclear cells were isolated from heparinized peripheral blood of healthy volunteers or from the patient by centrifugation on Ficoll/metrizoate (Lymphoprep; Nyegaard, Oslo) gradients and designated peripheral blood lymphocytes (PBLs). To isolate B and T cells, double rosetting of PBLs with neuraminidase-treated sheep erythrocytes was performed as described (21). Rosette-forming cells were designated T cells; non-rosetting cells, depleted of adherent cells on plastic Petri dishes for 30 min, were considered B cells.

Phenotypic Analysis of Lymphocytes. T and B lymphocytes and T-cell subsets were quantified in whole blood using monoclonal antibodies (mAbs) (Ortho-immune, Coulter clone, and Becton Dickinson reagents) by flow cytometry (22).

Detection of IL-2R. PBLs of the patient and a healthy control were cultured with and without phytohemagglutinin (PHA, 20 µg/ml) or concanavalin A (Con A, 8 µg/ml) for 24–48 hr and expression of IL-2R was examined by flow cytometry using anti CD-25 mAb (Becton Dickinson).

Lymphoproliferative Responses. The functional capacity of the patient's PBLs was assessed by measuring their proliferative responses to phytomitogens—namely, PHA, Con A, and pokeweed mitogen (PWM)—or common antigens (23). In certain experiments, a number of agents that are known to activate T cells (mAbs anti-CD2, anti-CD28, and anti-CD3; phorbol esters; and ionomycin) were used alone or in combination (24–30); rIL-2 was also used in an effort to augment responses. Responses were determined by measuring incorporation of [³H]thymidine or [¹⁴C]thymidine (New England Nuclear).

Assay for IL-2 Activity. PBLs (1×10^6) from the patient or a healthy control were cultured with or without PHA (20 µg/ml) or Con A (8 µg/ml) for 24–48 hr in culture medium with 10% fetal calf serum and 50 µM 2-mercaptoethanol in 5% CO₂/95% air. Supernatants were removed, filtered, and

Abbreviations: BMT, bone marrow transplantation; SCID, severe combined immunodeficiency disease; EBV, Epstein-Barr virus; IL-2, interleukin 2; IL-2R, IL-2 receptor(s); ISC, immunoglobulin-secreting cell(s); MLC, mixed lymphocyte culture; mAb, monoclonal antibody; PBL, peripheral blood lymphocyte; PHA, phytohemagglutinin; PMA, phorbol 12-myristate 13-acetate; PWM, pokeweed mitogen; rIL-2, recombinant human IL-2.

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frozen at -20°C until used for IL-2 assays. IL-2 activity in the supernatant was assayed by quantifying proliferation of cells of a murine IL-2-dependent cell line (HT-2). HT-2 cells were cultured with test supernatants for 24 hr, and 1.0 mCi of [³H]thymidine (1 Ci = 37 GBq) was added for the last 4 hr of culture. Cells were harvested to determine uptake of [³H]thymidine as described by Gillis (31) and Azoqui *et al.* (32).

Generation of Immunoglobulin Secreting Cells (ISC) *In Vitro*. Cultures consisting of 5×10^4 PBLs in 0.1 ml of RPMI 1640 medium supplemented with antibiotics and 15% fetal calf serum were set up in triplicate in round-bottomed microtiter plates (Costar). The cells were cultured for 7 days at 37°C in 5% CO₂/95% air in the presence or absence of the following stimuli: PWM, 10 µl/ml; heat-killed formalin-treated *Staphylococcus aureus* Cowan strain, 0.0015%; and Epstein-Barr virus (EBV)-containing culture supernatants derived from an infected B95-8 marmoset cell line, 150 µl/ml, as described (33). At the end of the culture period, cells were washed and assayed for ISC by reverse hemolytic plaque assay using protein A-coated sheep erythrocytes as targets and rabbit anti-human immunoglobulin as a developing serum.

Study Subject. Patient R.D. was born by caesarean section in December 1985, to a 30-yr-old white female after a 37-wk gestation. Birth weight was 2300 g. At age 2 mo, the patient presented with a diarrheal illness. Immediately thereafter, she developed vesicular lesions on the head, shoulders, and back after exposure to a sibling with chicken pox. Because the vesicular lesions persisted, R.D. was treated with acyclovir i.v. for 2 wk and recovered. Investigations to rule out immune deficiency showed a normal leukocyte count with no lymphopenia. Immunoglobulin levels in serum were low (IgG, 170 mg/dl, $n = 196-558$; IgA, <1 mg/dl, $n = 4-73$; IgM, 12 mg/dl, $n = 27-101$), and the patient failed to make adequate anti-varicella antibodies after onset of lesions characteristic of varicella. Although T- and B-cell numbers were normal, the patient had impaired B- and T-cell function *in vitro*. An enzymatic basis for immune deficiency was ruled out when adenosine deaminase and purine nucleoside phosphorylase were shown to be normal. A diagnosis of SCID was considered, and treatment with i.v. immunoglobulin was begun. The patient then developed *Pneumocytosis carinii* pneumonia, diagnosed by open lung biopsy, which was successfully treated with i.v. pentamidine after i.v. trimethoprim sulfamethoxazole failed to reverse the pulmonary disease. *S. aureus* cultured from a pustular infection of the thoracotomy site responded to oral dicloxacillin therapy. At 4 mo of age, the patient exhibited persistent thrush, which required continuous treatment with nystatin. She also exhibited failure to thrive, and at age 6 mo she was transferred to All Children's Hospital (Saint Petersburg, FL) for efforts to achieve immune reconstitution.

RESULTS

Immunological Assessment. Phenotypic analysis of lymphocytes. At age 6 mo, the patient had 84% CD3⁺ lympho-

cytes (T cells) and 9.8% CD20⁺ lymphocytes (B cells). Absolute numbers of T and B cells and the ratio of CD4⁺ T cells to CD8⁺ cells (T4/T8 ratio) were normal.

Lymphoproliferative responses and effect of exogenous IL-2. T-cell function assessed by thymidine uptake showed depressed lymphoproliferative response to PHA at various concentrations, but responses to Con A and PWM were within normal range. Lymphoproliferative responses to common antigens (*Candida* and tetanus) were markedly depressed (data not shown). Mixed lymphocyte culture (MLC) reactivity against three unrelated donors demonstrated 25% of the response seen with controls (data not shown).

To determine whether a lack of IL-2 might account for the unresponsiveness of the patient's PBLs to mitogens or to allogeneic cells, PBLs from the patient and from a healthy control were cultured with optimal concentrations of PHA, Con A, and PWM in the presence of rIL-2. As shown in Table 1, the patient's depressed responses to PHA were augmented by the addition of as little as 1 unit of exogenous rIL-2 per ml. rIL-2 also enhanced the responses of patient cells in MLC reactions (data not shown).

The functional defect in T-cell lymphoproliferation was further investigated by activating patient T cells with mAb directed against CD3 and CD2 receptors (24-30) and with a combination of phorbol 12-myristate 13-acetate (PMA) and ionomycin (30). Depressed lymphoproliferative responses to anti-CD2 and anti-CD3 mAbs were augmented by the addition of rIL-2 to the cultures. The patient's cells also proliferated when cultured with a combination of PMA and ionomycin or with PMA and anti-CD2 mAb.

Induction of IL-2R and IL-2 secretion. When the patient's PBLs were cultured with PHA or Con A, expression of IL-2R increased from <1% to 12% and 20%, respectively. However, the patient's cells failed to secrete IL-2 after stimulation with PHA or Con A (Fig. 1). In another set of experiments, the patient's cells secreted low amounts of IL-2 in response to a combination of PMA and ionomycin (data not shown).

Analysis of DNA and mRNA for IL-2. Although the IL-2 gene was normal in Southern blot analysis, induction of mRNA for IL-2 was absent, suggesting that the abnormality in IL-2 secretion was linked to a failure of IL-2 gene transcription (34, 35).

In vitro studies of B-cell differentiation. The patient's B cells showed poor ISC response to three stimuli (PWM, EBV, and *S. aureus* Cowan strain) as compared to normal controls (data not shown). That the patient's B cells were intrinsically defective is suggested by the observation (Table 2) that purified patient B cells failed to respond to EBV or to PWM in the presence of normal T cells. Surprisingly, the patient's T cells could provide adequate help to normal B cells in their differentiation response to PWM. Exogenous IL-2 failed to significantly influence ISC response of patient or control PBLs to any stimuli (data not shown).

Clinical Course and Treatment. Treatment with BMT. At age 6 mo, the child was hospitalized, isolated by laminar air flow, and given oral nonabsorbable antibiotics and total

Table 1. Lymphoproliferative responses, IL-2R expression, and *in vitro* effect of rIL-2

Stimulus	Patient			Control		
	Cells	Cells + IL-2	IL-2R expression % positive	Cells	Cells + IL-2	IL-2R expression % positive
PHA	1735	38,547	12.0	21,041	25,603	32.6
Con A	3989	23,579	20.8	4,710	8,753	38.1
PWM	4978	5,171	ND	4,501	4,695	ND
Medium	177	571	0.1	353	1,246	0.2

Lymphoproliferative responses were measured by ¹⁴C uptake (cpm). After patient or control PBLs were cultured with rIL-2 (Cetus; 1 unit/ml) for 40 hr, they were stained with anti-IL-2 receptor antibody (Becton Dickinson). ND, not done.

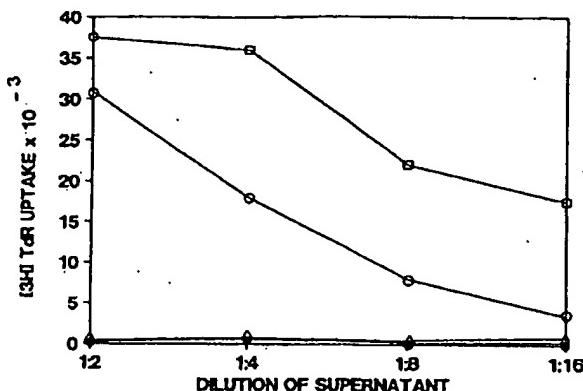


FIG. 1. PBLs (1×10^6) from patient R.D. and from a healthy control were stimulated with PHA ($10 \mu\text{g}/\text{ml}$) (●, patient; □, control) or Con A (△, patient; ○, control) for 48 hr. As measured by [^3H]thymidine uptake, the patient's cells failed to secrete IL-2 in response to stimulation.

parenteral nutrition. Since an HLA-identical MLC-nonreactive matched sibling donor was not available, an attempt was made to correct the patient's immunodeficiency disease by a T-depleted bone marrow graft from a haploidentical paternal donor. BMT was done after extensive myeloablation with busulfan ($4 \text{ mg kg}^{-1}\text{day}^{-1}$ for 4 days) and immunosuppression with Cytoxan (cyclophosphamide) ($50 \text{ mg kg}^{-1}\text{day}^{-1}$ for 4 days). Initially the graft was accepted, but subsequently it failed or was rejected 50 days after BMT. A second T-cell-depleted BMT given as a booster 81 days later also was rejected.

Treatment rIL-2. After failure of the BMT, the long-term prognosis for this patient was very poor. Since studies had revealed normal expression of IL-2R but deficiencies of IL-2 production by R.D.'s T lymphocytes, a therapeutic trial was undertaken to administer rIL-2 in incremental doses (Fig. 2). Therapy began on day 339 of hospitalization, with $10,000 \text{ units kg}^{-1}\text{day}^{-1}$ of rIL-2 administered by continuous i.v. infusion over a period of 6 hr. After 2 wk, the dose was increased to $20,000 \text{ units kg}^{-1}\text{day}^{-1}$ for 7 days and then to $30,000 \text{ units kg}^{-1}\text{day}^{-1}$ i.v. for 2 wk. The child tolerated these infusions well. Dosage was then increased to $40,000 \text{ units kg}^{-1}\text{day}^{-1}$ i.v., to be infused over a 6-hr period. With the first infusion at this dose, the patient exhibited hyper-

tension (blood pressure, $140/94 \text{ mmHg}$) and tachycardia (heart rate, 140–160 beats per min). The infusion was stopped, and blood pressure and pulse stabilized within 8 hr. When the same high dose was administered the next day, a similar hemodynamic reaction occurred. This reaction was deemed a side effect of the rIL-2 therapy, and thus the dosage was reduced to $30,000 \text{ units kg}^{-1}\text{day}^{-1}$. On day 441 of hospitalization, this dosage was reduced in frequency to three times weekly (Monday, Wednesday, and Friday) by i.v. infusion over a period of 1 hr. The patient tolerated this regimen well and was discharged from the hospital on day 464.

Effect of therapy on lymphoproliferative responses. Fig. 2 shows the lymphoproliferative responses to PHA, Con A, and PWM during the course of BMT and subsequent IL-2 therapy. On day 0 (date of hospitalization), the patient showed depressed proliferative responses to PHA but normal responses to Con A and PWM. The first BMT was done on day 159 of hospitalization; the second was done on day 240. After BMT, the lymphoproliferative responses to PHA, Con A, and PWM were extremely low from day 159 to day 339. When IL-2 therapy was begun, proliferative responses increased exponentially and became maximal at a dose of $30,000 \text{ units/kg}$. After rIL-2 therapy was temporarily discontinued on day 397 for 3 wk, proliferative responses to PHA, Con A, and PWM decreased by >50% within a week. When therapy was resumed on day 422, proliferative responses to Con A and PWM normalized, and PHA responses were frequently, but not always, within the normal range.

Effect of therapy on lymphocyte phenotypes. Upon admission (day 0) at age 6 mo, the patient had normal proportions and numbers of T (84.0%) and B (9.8%) cells. After BMT, from days 159–308 of hospitalization, the percentage of T cells became low, ranging from 4% to 20%, and the number of B cells increased, ranging from 3% to 54%. After initiation of rIL-2 therapy (day 339), the percentage of T cells gradually increased from 37.5% to 84.0%, and the proportion of B cells decreased from 34.0% to 11.0%. These results indicate that *in vivo* rIL-2 therapy served to normalize the total numbers and proportions of T and B cells.

Current clinical status. Since her discharge from the hospital in September 1987, the patient has been receiving rIL-2 therapy at home at a dosage of $30,000 \text{ units/kg}$ i.v. three times weekly via a broviac catheter. She remains free of serious infections and has not required hospitalization. At age 3 yr, she is just below the 50th percentile for height and weight.

DISCUSSION

In this study, we describe a patient with a primary defect in IL-2 secretion who has been managed successfully with rIL-2 therapy. The clinical illness was compatible with a diagnosis of SCID featured by persistent thrush, recurrent infections, persistent viral infections, and opportunistic infection with *P. carinii* pneumonia. The patient had phenotypically normal T- and B-lymphocyte distribution, but studies on T-lymphocyte activation via lectins demonstrated defective lymphoproliferative responses to PHA and responses at the low part of the normal range to Con A and PWM. Defective lymphoproliferative responses to allogeneic cells, common antigens, and anti-CD3 and anti-CD2 mAbs were also observed. Exogenous IL-2 consistently augmented lymphoproliferative responses to PHA, PMA, and anti-CD2 and anti-CD3 mAbs.

In the case of resting T cells, macrophages and/or IL-2 are essential for the initial responsiveness of CD3Ti receptor triggering (CD3 pathway) and to phytomitogens (36), whereas IL-1 and/or monocytes are not required for activation of the T cells via the CD2 pathway (30, 37). In this patient, IL-1 or normal irradiated monocytes failed to reconstitute the patient's low lymphoproliferative responses to phytomitogens

Table 2. Evidence for an intrinsic B-cell defect in patient R.D.

Culture	ISC $\times 10^{-3}$ per 10^6 cultured cells
PWM stimulation	
Patient B + patient T	7.5
Patient B + patient T*	10.2
Patient B + patient T	3.8
Patient B + control T	13.4
Control B + control T	71.8
Control B + patient T	58.5
Control B + patient T*	62.2
Control B + patient T	38.2
EBV stimulation	
Patient B	7.8
Control B	47.5

Purified B cells from patient R.D. failed to respond to stimulation with EBV or PWM in the presence of either patient or control T cells, suggesting an intrinsic B-cell defect. Additions of B and T cells were done at a 1:1 ratio; final cell concentration, 5×10^4 cells per well. Background ISC counts of B or T cells cultured alone were <0.1. *Irradiated T cells (2000 rads; 1 rad = 0.01 Gy).

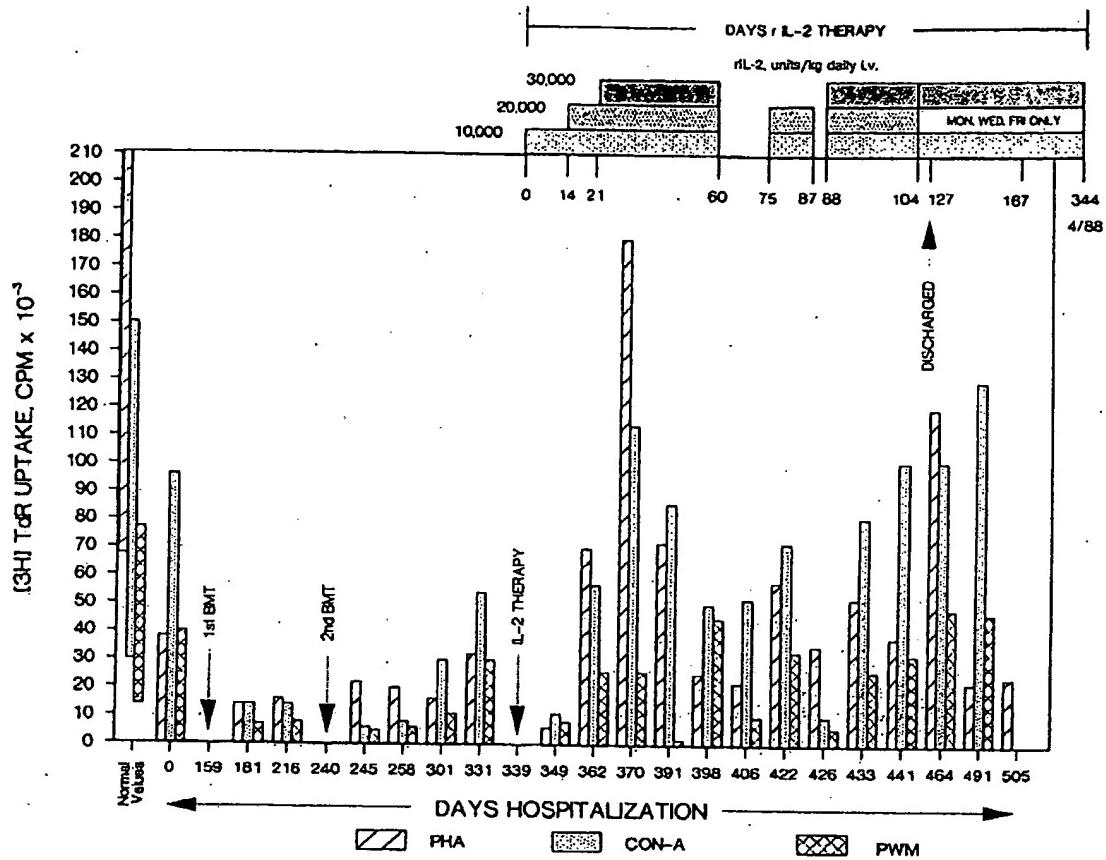


FIG. 2. Clinical course and lymphoproliferative responses. Vertical bars represent lymphoproliferative responses to phytohemagglutinins. Horizontal bars indicate periods of IL-2 therapy at the doses indicated.

(data not shown). Furthermore, the patient's irradiated monocytes could support the proliferative responses of normal T cells, ruling out a primary defect of either monocyte-supportive function or IL-1 production. The observed enhancement of lymphoproliferative responses with exogenous rIL-2 suggested that the abnormalities observed in this patient might be linked to expression of IL-2R or to IL-2 production. The patient's cells failed to secrete IL-2 after stimulation with different lectins, but the cells did secrete a minimal amount of IL-2 after combined stimulation with PMA and ionomycin. Analysis at the molecular level showed no mRNA for IL-2 in a sample from the patient after PHA stimulation, suggesting that this patient's deficient expression of mRNA was linked to a failure to transcribe DNA that encoded secretion of IL-2 (T.C., E. Castigalli, R.P., R.A.G., and R.G., unpublished data).

Recent work (38, 39) suggests that IL-2 production depends on two early activation signals that are initiated by T-cell membrane perturbation: an increase in intracellular Ca^{2+} and activation of protein kinase C. The activation of CD3 ζ receptors results in the cleavage of phosphatidylinositol 4,5-bisphosphate to inositol 1,4,5-trisphosphate (InsP_3) and diacylglycerol. InsP_3 causes release of Ca^{2+} from intracellular stores, while diacylglycerol activates protein kinase C. These two signals then synergize to initiate transcription of the IL-2 gene. Given that IL-2 mRNA was absent in our patient, it is possible that the patient's cellular defect may reside in inositol phospholipid metabolism leading to unresponsiveness of the T cells; such could be the case even

though some T-cell activation events could occur—e.g., partial IL-2R and some degree of response to Con A and PWM (38, 39). Alternatively, T-cell proliferation in this patient may have been possible via an interleukin-independent pathway for lymphocyte activation (40).

The patient described here also appears to have an intrinsic defect of the B lymphocytes, as demonstrated by a defect in the differentiation of B cells into ISC. This abnormality, which occurs commonly in patients with SCID, was not corrected by addition of normal T cells and/or by exposure of B cells to exogenous IL-2. It is interesting to note that the patient's T cells could provide adequate help to normal B cells. Since production of IL-2, IL-4, and IL-6 is independently regulated (41), and since each is important in B-cell differentiation and immune response, it may be that production of the latter two factors is intact in this patient.

Recently, several patients with the SCID phenotype who have a defect in T-lymphocyte activation have been described. Such defects include the absence of IL-1 production (42) and defects of IL-2R expression and of IL-2 production (13–20). IL-2 receptors are expressed by activated T lymphocytes, and the binding of this receptor to its ligand is required for T-cell proliferation. Our findings imply that defective IL-2 secretion also results in one form of the SCID phenotype, which as reported herein has several distinct features. Such patients may have normal numbers of T and B cells based on surface markers and may manifest various degrees of deficiency in the T-cell activation pathways related to CD2 and CD3 stimulation and diminished prolifera-

tive responses to mitogens and antigens, all of which can be consistently augmented *in vitro* by rIL-2. In the patient described here, appropriately stimulated lymphocytes expressed the 55-kDa chain of the IL-2R but did not secrete IL-2. It is not known whether the patient could express the 75-kDa chain of the IL-2R. A likely defect in this patient's cells is a defect at the mRNA level, which is responsible for transcription of DNA that encodes IL-2 (T.C., E. Castigalli, R.P., R.A.G., and R.G., unpublished data).

In efforts to treat patient R.D., two haploidentical BMTs were unsuccessful. It is possible that engraftment of IL-2-producing donor cells allowed residual recipient T lymphocytes to proliferate and destroy the histoincompatible donor cells. With the failure of BMT, the long-term prognosis for this patient appeared very poor. At this point, treatment was initiated with rIL-2 (10,000 units/kg) given daily i.v. and was gradually increased to 30,000 units/kg i.v. This dosage of rIL-2 was well tolerated, as documented by clinical and laboratory analyses. The success of this new therapeutic approach underscores the need to establish the etiology of SCID in each affected patient and points to another direction for treatment by using rIL-2 for certain SCID patients.

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Human Invariant NKT Cells Are Required for Effective In Vitro Alloresponses¹

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NKT cells are a small subset of regulatory T cells conserved in humans and mice. In humans they express the $\text{V}\alpha 24\text{J}\alpha 18$ invariant chain (hence invariant NKT (iNKT) cells) and are restricted by the glycolipid-presenting molecule CD1d. In mice, iNKT cells may enhance or inhibit anti-infectious and antitumor T cell responses but suppress autoimmune and alloreactive responses. We postulated that iNKT cells might also modulate human alloreactive responses. Using MLR assays we demonstrate that in the presence of the CD1d-presented glycolipid α -galactosylceramide (α GC) alloreactivity is enhanced ($37 \pm 12\%$; $p < 0.001$) in an iNKT cell-dependent manner. iNKT cells are activated early during the course of the MLR, presumably by natural ligands. In MLR performed without exogenous ligands, depletion of iNKT cells significantly diminished the alloresponse in terms of proliferation ($58.8 \pm 24\%$; $p < 0.001$) and IFN- γ secretion ($43.2 \pm 15.3\%$; $p < 0.001$). Importantly, adding back fresh iNKT cells restored the reactivity of iNKT cell-depleted MLR to near baseline levels. CD1d-blocking mAbs equally reduced the reactivity of the iNKT cell-replete and -depleted MLR compared with IgG control, indicating that the effect of iNKT cells in the in vitro alloresponse is CD1d-dependent. These findings suggest that human iNKT cells, although not essential for its development, can enhance the alloreactive response. *The Journal of Immunology*, 2005, 175: 5037–5044.

Natural killer T cells are a small but potent subset of regulatory T cells highly conserved in humans and mice (1). Human NKT cells comprise <0.1% of blood T cells and are characterized by a unique TCR made up of an invariant $\text{V}\alpha 24\text{J}\alpha 18$ ($\text{V}\alpha 14\text{J}\alpha 18$ in mice) and a diverse $\text{V}\beta 11$ chain (2), hence the term invariant NKT (iNKT)² cells. iNKT cells also express the NK cell marker CD161 (NK1.1 in mice); however, its expression varies with the activation status of the cell (1, 3). In humans only a small proportion of CD161⁺ T cells are iNKT cells, whereas in mice ~50% of NK1.1⁺ T cells are iNKT cells as identified by CD1d/ α -galactosylceramide (α GC) tetramer staining (1, 4). Upon engagement of their TCR by the glycolipid-presenting MHC class I-like molecule CD1d, iNKT cells are rapidly activated and secrete large amounts of Th1 and Th2 cytokines (1). α GC, a marine sponge glycolipid presented by CD1d, potently activates iNKT cells in vitro as well as in vivo (1, 5). Activated iNKT cells play a pivotal role in modulating all aspects of the innate and adaptive immune responses mainly through interactions with APCs (1). For example, in response to α GC, iNKT cells regulate Ag-specific in vivo Th1 cell responses by increasing dendritic cell

(DC) expression of surface molecules important in Ag presentation such as HLA, CD80, CD83, and CD86 (6, 7). NKT cell activation often leads to enhanced responses against pathogens (e.g., viruses) and tumors and suppression of autoimmunity (reviewed in Refs. 1, 8, and 9). However, depending on the experimental context, NKT cell activation may also be associated with increased susceptibility to viral disease and loss of tumor immunosurveillance (reviewed in Refs. 1, 8, and 9).

Alloreactivity, one of the most powerful immune responses, is a Th1 cell- and cytokine-mediated response directed against disparate major or minor histocompatibility Ags. As in other types of immune responses, it is likely that the magnitude and the quality of the alloreactive response is modulated by networks of regulatory T cells that may enhance or dampen the effects of cytokine and cell allofactors. Understanding these networks may offer a basis for development of rational and novel therapeutic approaches for the control of the alloresponse in the clinical arena.

The role of iNKT cells in the modulation of alloreactivity has been studied in murine models of acute graft-vs-host disease (GVHD). Host iNKT cells appear to protect from lethal acute GVHD in systems involving myeloablative (10–12) as well as nonmyeloablative host preparation protocols (13–15). In addition, bone marrow-derived donor NKT cells as identified by expression of NK1.1 (or CD161 in humans) suppress the alloresponse (16, 17).

The modulatory effect of human NKT cells, as they are defined by the expression of the invariant $\text{V}\alpha 24\text{J}\alpha 18$ chain, in the alloreactive response has not been studied. In this study, using MLR assays, we dissect the role of human iNKT cells, α GC, and CD1d in the modulation of the in vitro alloresponse.

Materials and Methods

CD3⁺ T cell selection

Buffy coats from normal blood donors were supplied by the North London Blood Transfusion Service under Local Research Ethics Committee approval. PBMC were obtained after layering over Ficoll. For selection of

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⁴Abbreviations used in this paper: iNKT, invariant NKT; α GC, α -galactosylceramide; DC, dendritic cell; GVHD, graft-vs-host disease.

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CD3^+ cells, the EasySep negative selection kit was used as per manufacturer's instructions (StemCell Technologies). Purity of CD3^+ cells was always >90% (data not shown).

Flow cytometry and flow sorting

Cells were stained with mAbs using standard protocols (18). For the identification and flow sorting of iNKT cells, selected CD3^+ cells were stained with mAb against TCR $\text{V}\alpha 24$ and $\text{V}\beta 11$. With this approach the vast majority but not all bona fide iNKT cells as defined by $\text{CD1d}/\alpha\text{GC}$ tetramer staining are identified (18, 19). Therefore, depletion of iNKT cells using TCR $\text{V}\alpha 24$ and $\text{V}\beta 11$ mAb will remove most $\text{CD1d}/\alpha\text{GC}$ tetramer-positive iNKT cells except a very small population of $\text{CD1d}/\alpha\text{GC}$ tetramer-positive TCR $\text{V}\alpha 24$ -negative iNKT cells (19). The following mAbs were used: mouse anti-human TCR $\text{V}\alpha 24$ and TCR $\text{V}\beta 11$ either FITC or RPE-labeled, or biotin-labeled (Serotec); mouse anti-human biotin (Beckman Coulter); CD69-FITC, CD3-allophycocyanin, IgG1-FITC, IgG1-RPE, and IgG1-biotin (Cahag Laboratories); and CD44-PeCP, HLA-DR-allophycocyanin, and streptavidin-allophycocyanin (BD Biosciences). Multicolor

flow cytometry was performed using a FACSCalibur, whereas flow sorting was performed using a FACSDiva (BD Biosciences). Data analysis was performed with the CellPro or FlowJo software.

MLR and proliferation assays

For all cultures, T cell medium consisting of RPMI 1640, 5% heat-inactivated human serum supplemented with 1% of L-glutamine and penicillin/streptomycin was used. For MLR, generally $30-50 \times 10^3 \text{ CD3}^+$ cells were placed in triplicates against autologous or allogeneic irradiated (3000 rad) PBMC in 96-well plates at responder to stimulator (R:S) ratios as indicated. For long-term MLR, $30-50 \times 10^3 \text{ CD3}^+$ cells were plated in 48-well plates. [^3H]Thymidine was added at 1 $\mu\text{Ci}/\text{well}$ for the last 16 h of the MLR. Proliferation was measured using a liquid scintillation counter after harvesting with a cell harvester. Parallel cultures were set for cytokine release assay. In indicated experiments, oGC (100 ng/ml) or its diluent vehicle (NaCl 150 mM/Tween 20 0.03%) were added to the MLR. The following mAbs were used for blocking MLR: anti-CD1d (clone 42.1; BD

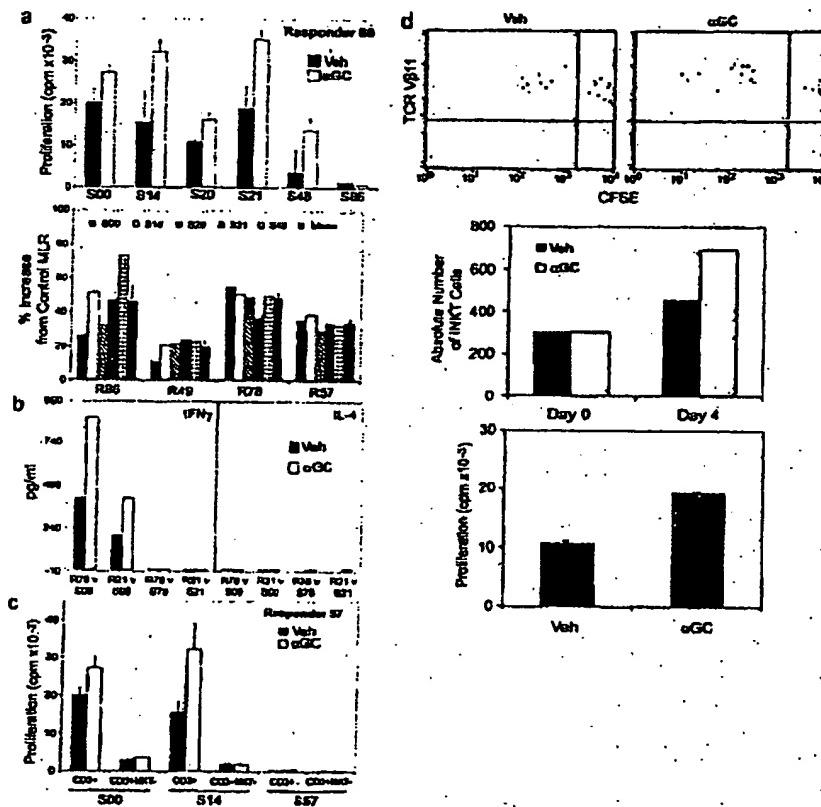


FIGURE 1. oGC enhances alloreactivity in an iNKT cell-dependent manner. *a*, MLR was performed using T cells from responder 86 against a panel of five stimulators in the presence of vehicle or oGC (top). In the presence of oGC, proliferation increased by 48% (range, from 25 to 73%). Minimal reactivity was observed in autologous MLR. In total, MLR of four responders against five stimulators (each repeated twice) were tested and in all cases addition of oGC (bottom) had an enhancing effect on the alloreactive response ($37 \pm 12\%$; $p < 0.001$). *b*, IFN- γ production was increased in oGC-treated alloigenic compared with vehicle-treated MLR but not in autologous MLR. No IL-4 production was detected under these conditions. Data are representative of four independent MLR. *c*, iNKT cell-deplete or cell-depleted MLR were performed in the presence of vehicle or oGC. In iNKT cell-deplete allo-MLR, proliferation was equally reduced under both conditions, indicating that the enhancing effect of oGC on the alloresponse is iNKT cell-dependent (CD3^+ indicates iNKT cell-deplete MLR, and $\text{CD3}^+\text{NKT}^-$ indicates iNKT cell-depleted MLR). *d*, Proliferation as determined by CFSE staining (upper) and absolute numbers (middle) of iNKT cells (as determined by flow cytometry and total cell count) in a 96-h MLR in the presence of oGC or vehicle. In parallel, proliferation was measured by [^3H] incorporation (lower). Blots are gated on iNKT cells identified by anti-TCR $\text{V}\alpha 24$ and $\text{V}\beta 11$ staining. iNKT cells proliferate more in the presence of oGC; however, their increase in absolute numbers does not account for the 50% increase in proliferation observed in the presence of oGC. Data are representative of two independent experiments and are presented as mean \pm SEM of triplicate assays.

Pharmingen); anti-HLA class II (clone TU39; BD Pharmingen); and IgG1 isotype (BD Pharmingen) at indicated concentrations.

CFSE (Molecular Probes) staining of CD3⁺ cells was performed in PBS for 8 min at room temperature using CFSE at 5 μ M with occasional mixing. After washing with an equal volume of FCS, cells were extensively washed with complete T cell media.

ELISA

Quantitation of IFN- γ and IL-4 in the supernatants of the MLR was performed with the Quantikine kit (R&D Systems).

Statistical analysis

The Wilcoxon signed ranks test was used to compare differences in proliferation between iNKT cell-deplete and cell-depleted MLR and between α GC or vehicle-treated MLR.

Results

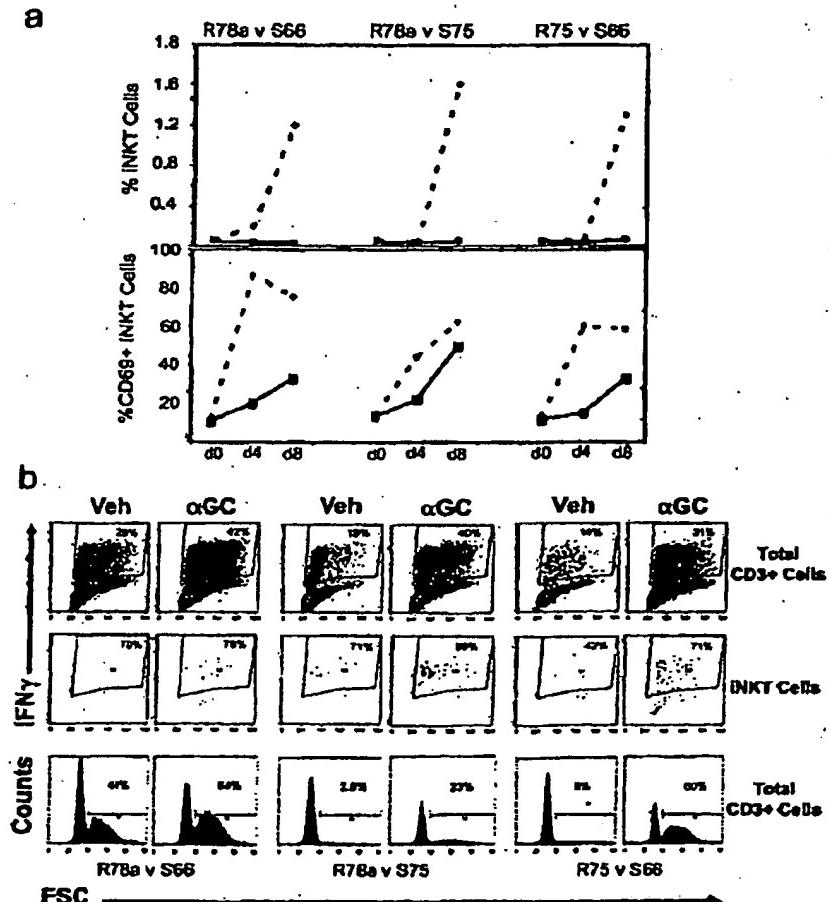
Invariant NKT cell-dependent enhancement of MLR by α GC

In *in vivo* murine models, treatment of irradiated recipient mice with α GC induces host-derived, iNKT cell-dependent IL-4 secretion and protection from acute GVHD (11, 12). We tested the effect of α GC in human *in vitro* alloresponses. For this purpose we performed 4-day MLR, using as responders purified negatively selected T cells and as stimulators irradiated PBMC pulsed with

α GC or vehicle. As shown in Fig. 1a, top, addition of α GC to the MLR of responder 86 against a panel of five stimulators caused a 25–73% increase in proliferation. To rule out nonspecific T cell activation that may be induced by α GC (20), autologous MLR was performed in parallel with the allogeneic MLR. In the presence of either α GC or vehicle, minimal proliferation was observed in autologous as compared with allogeneic MLR (Fig. 1a, top), suggesting thus that the enhancing effect of α GC in the allogeneic MLR is alloreactive T cell-specific. In addition to responder 86, we tested another three responders against a panel of five stimulators and found that treatment of the MLR with α GC resulted in a 37 \pm 12% ($p < 0.001$) increase in proliferation as compared with MLR treated with vehicle only (Fig. 1a, bottom). Consistent with this, IFN- γ production was also comparably increased in the MLR performed in the presence of α GC; no IL-4 was detected at 96 h (Fig. 1b) or at 4 and 24 h of the MLR (data not shown). Furthermore, the enhancing effect of α GC was iNKT cell-dependent, as upon iNKT cell depletion (see below) and in the presence of α GC, proliferation was equally affected as in iNKT cell-depleted MLR treated with vehicle (Fig. 1c).

To further elucidate the relative role of iNKT cells in the enhancement of MLR by α GC, we correlated proliferation of iNKT

FIGURE 2. Expansion and activation of iNKT cells in long-term MLR. *a*, Three independent MLR were performed in the presence of α GC or vehicle without exogenous IL-2 supplementation. On indicated time points the frequency as well as the activation of iNKT cells was determined by flow cytometry after staining with anti-TCR V α 24, V β 11, and CD69. At least 5×10^6 events were collected for analysis. The frequency of iNKT cells in the MLR treated with vehicle (top, solid line) remained similar to baseline throughout the course of the MLR. In the presence of α GC (dashed line), the frequency of iNKT cells on day 4 was similar to baseline; however, it increased by at least 10-fold by day 8. The frequency of naturally activated iNKT cells progressively increases during the course of the MLR (bottom). Greater activation of iNKT cells is seen when cells are pharmacologically activated by exogenous α GC. *b*, IFN- γ production by iNKT cells and non-iNKT T cells in MLR treated with α GC or vehicle on day 8. α GC induces more IFN- γ production by total T cells as well as iNKT cells. In addition, activation of non-iNKT cells as assessed by forward scatter characteristics is also higher in the presence of α GC. Gates for iNKT cells have been set on TCR V α 24⁺V β 11⁺ events. Gates for total CD3 cells for both blots and histograms exclude iNKT cells.



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cells (as assessed by CFSE labeling) and absolute counts (as determined by flow cytometry and total cell count) with the proliferation of all T cells (as assessed by ^3H incorporation). CFSE staining showed that iNKT cells proliferated more in the presence of αGC compared with vehicle (Fig. 1d, upper). This proliferation corresponded to an absolute increase from a baseline of 300 iNKT cells on day 0 (corresponding to 2×10^6 plated total T cells) to 450 and 690 cells 4 days later in the presence of vehicle and αGC , respectively (Fig. 1d, middle). At the same time, the MLR reactivity of total T cells (5×10^6 responder T cells corresponding to $\sim 10-15$ iNKT cells) increased by almost 50% (corresponding to $\sim 10,000$ cpm) in the presence of αGC (Fig. 1d, lower). It is very unlikely that this difference is the result of proliferation of the few NKT cells activated on day 4 by αGC ; instead, it reflects the proliferation of the alloreactive T cells.

Dynamics of iNKT cell activation in MLR

αGC is a pharmacological agent and its effect on iNKT cell expansion and activation may not reflect the mechanisms of iNKT

cell activation by natural CD1d ligands. The dynamics of iNKT cell expansion and activation (as determined by the surface activation marker CD69) in response to natural ligands were monitored during the course of long-term MLR (Fig. 2). In the presence of αGC the relative frequency of iNKT cells did not increase significantly on day 4, but it did increase by at least 10-fold by day 8 (Fig. 2a, top). As well as proliferation, αGC also induced increasing activation of iNKT cells over time (Fig. 2a, bottom). The lack of relative expansion of αGC -treated iNKT cells on day 4 might be more apparent rather than real because upon αGC exposure, the TCR of iNKT cells is known to be initially down-regulated but re-expressed later [21, 22]. In the presence of vehicle, the relative frequency of iNKT cells during the 8-day MLR did not change significantly from baseline (Fig. 2a, top). Despite the lack of significant proliferation, vehicle-treated iNKT cells were progressively activated between days 0, 4, and 8 of the MLR (Fig. 2a, bottom). iNKT cells therefore are activated during the course of the MLR in the absence of αGC , presumably by natural ligands presented to them by CD1d-expressing APC, although they do not

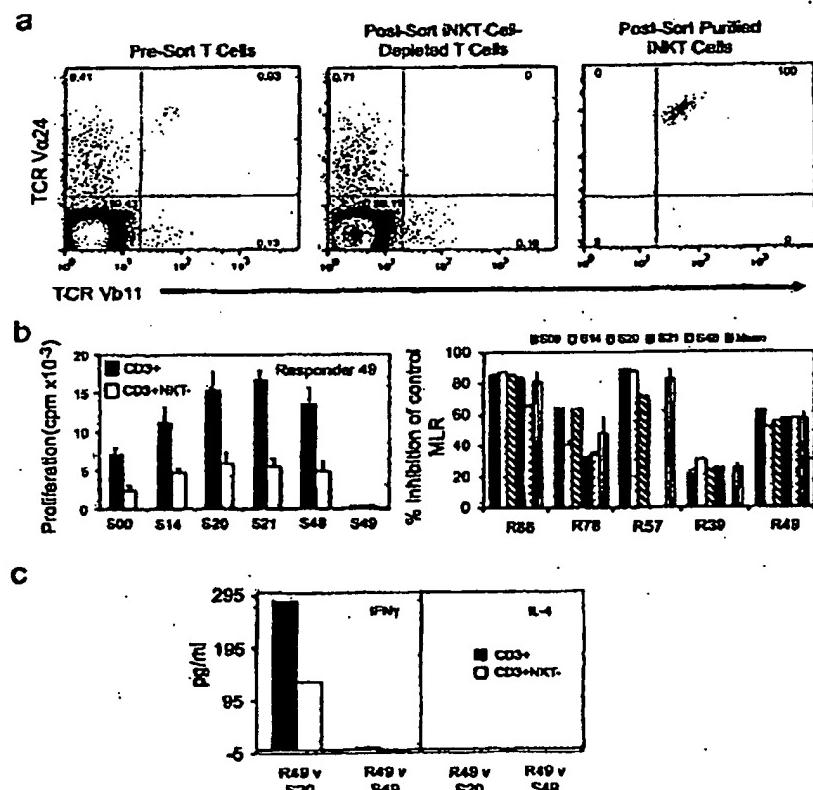


FIGURE 3. iNKT cell depletion attenuates in vitro alloresponse. *a*, iNKT cell depletion of CD3⁺ T cells by flow sorting. Negatively selected CD3⁺ cells with a purity of >90% (data not shown) were stained with anti-TCR Va24 and Vb11 mAbs. Using a FACSDiva flow sorter, TCR Va24⁺Vb11⁺ NKT cells were depleted from CD3⁺ cells. Pre- and postsorting analysis of at least 5×10^6 events revealed that the procedure consistently generated highly iNKT cell-depleted CD3⁺ cells and highly purified iNKT cells. In addition, CD3⁺ iNKT cell-deplete cells were sorted on the basis of their physical characteristics (data not shown). *b*, iNKT cell-deplete MLR (indicated as CD3⁺) using T cells from responder 49 against a panel of five stimulators was compared with iNKT cell-depleted MLR (indicated as CD3+NKT⁻) (left). In total, MLR of four responders were tested against three to five stimulators (each panel repeated twice with similar results), and in every responder to stimulator pair, iNKT cell depletion significantly diminished the alloreactive response ranging from 36 to 85% ($p < 0.001$; right). Data are presented as mean \pm SEM of triplicate assays. *c*, in accordance with the proliferation data, IFN- γ secretion was comparably reduced in iNKT cell-depleted MLR compared with baseline. No IL-4 was detected (representative of seven independent MLR).

proliferate significantly. On day 8 of the same series of MLR, we studied IFN- γ production by iNKT cells as well as total T cells. IFN- γ production was higher in iNKT cells treated with α GC rather than vehicle and, importantly, IFN- γ production by total T cells was considerably higher in the presence of α GC (Fig. 2b). In addition, in the presence of α GC there was increased activation of non-iNKT cells as determined by forward scatter characteristics (Fig. 2b) and CD69 expression (data not shown). These findings provide further evidence that increased reactivity of MLR in the presence of α GC does not merely reflect activation of iNKT cells but is primarily due to activation of alloreactive T cells.

Role of iNKT cells in the absence of exogenous ligand

The findings discussed above suggest that human iNKT cells are an integral part of the *in vitro* alloresponse and are required for its efficient development. This predicts that depletion of iNKT cells would attenuate the alloreactivity. To test this, we performed 4-day MLR using either iNKT cell-replete or cell-depleted responders. Rigorous iNKT cell depletion of negatively selected T cells was performed by staining with anti-TCR V α 24 and V β 11 mAbs followed by flow sorting (Fig. 3a). iNKT cell-replete T cells were also flow sorted on the basis of their physical characteristics. To rule out preactivation of iNKT cells triggered by staining with the anti-TCR V α 24 and V β 11 mAbs we established that engagement of the invariant TCR by the anti-TCR mAbs during the sorting procedure did not have mitogenic effect on iNKT cells and did not alter their Th1/Th2 cytokine secretion balance (data not shown). When iNKT cells were depleted, the MLR reactivity of responder 49 against a panel of stimulators decreased by ~65% (Fig. 3b, left). In an extended panel in which we tested another three responders against three to five stimulators, the proliferation rate in iNKT cell-depleted compared with iNKT cell-replete MLR was reduced (by $58.8 \pm 24\%$; $p < 0.001$) in every responder to stimulator pair (Fig. 3b, right). This suppressive effect was mirrored by a comparable reduction of IFN- γ secretion in the supernatants of the iNKT cell-depleted MLR compared with baseline (by $43.2 \pm 15.2\%$; $n = 7$; $p < 0.001$) (Fig. 3c). No IL-4 was detected. The effect of iNKT cell depletion was observed in MLR performed with varying R:S ratios (Fig. 4a) and also in longitudinal MLR (Fig. 4b). Finally, add-back of highly purified fresh iNKT cells to iNKT cell-depleted MLR restored proliferation close to the levels of the baseline iNKT cell-replete MLR (Fig. 4c). These findings suggest that although iNKT cells are not essential, they do contribute to the development of full MLR reactivity.

Role of CD1d in MLR

CD1d is the restricting element of iNKT cells and is expressed on monocytes, B cells, and APC. We therefore tested the effect of anti-CD1d mAb on the MLR. Anti-CD1d caused a dose-dependent inhibition of MLR as compared with Ig isotypic control (Fig. 5a) and anti-CD1d used at a fixed dose inhibited MLR at different R:S ratios (Fig. 5b). In longitudinal MLR, T cell proliferation and activation as determined by CFSE and HLA-DR staining, respectively, were significantly and dynamically decreased in the presence of anti-CD1d compared with Ig isotypic control (Fig. 5c). Further, T cells stimulated in primary MLR in the presence of anti-CD1d were less responsive in secondary MLR as compared with T cells derived from primary MLR treated with isotypic Ig control (Fig. 5d), suggesting that CD1d blocking may have rendered responder T cells anergic.

CD1d is required for the iNKT cell-mediated effect on MLR

In principle, the effect of anti-CD1d mAb on MLR might reflect either inhibition of an enhancing effect of NKT cells on the MLR

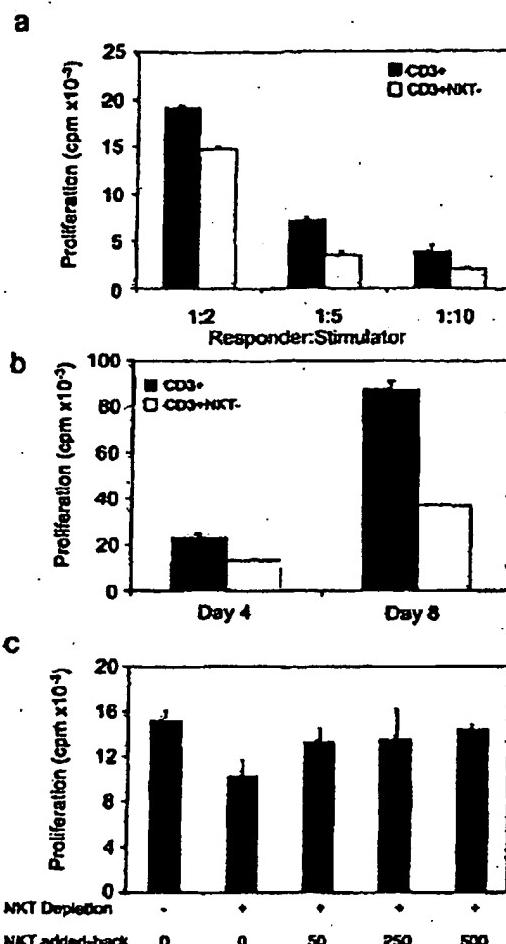


FIGURE 4. Dynamics of the alloresponse inhibition by iNKT cell depletion. *a*, iNKT cell depletion reduces MLR reactivity at varying R:S ratios and at different time points and a fixed R:S ratio of 1:2 (*b*). *c*, iNKT cells were *ex vivo* purified by flow sorting as shown in Fig. 3*a* and were used in an add-back MLR assay. Addition of 50 responder iNKT cells (corresponding roughly to the number of responder iNKT cells in the iNKT cell-replete MLR) were added to iNKT cell-depleted MLR. MLR reactivity increased by >50% and even further when 5- and 10-fold more iNKT cells were added back. Each result is representative of three independent MLR.

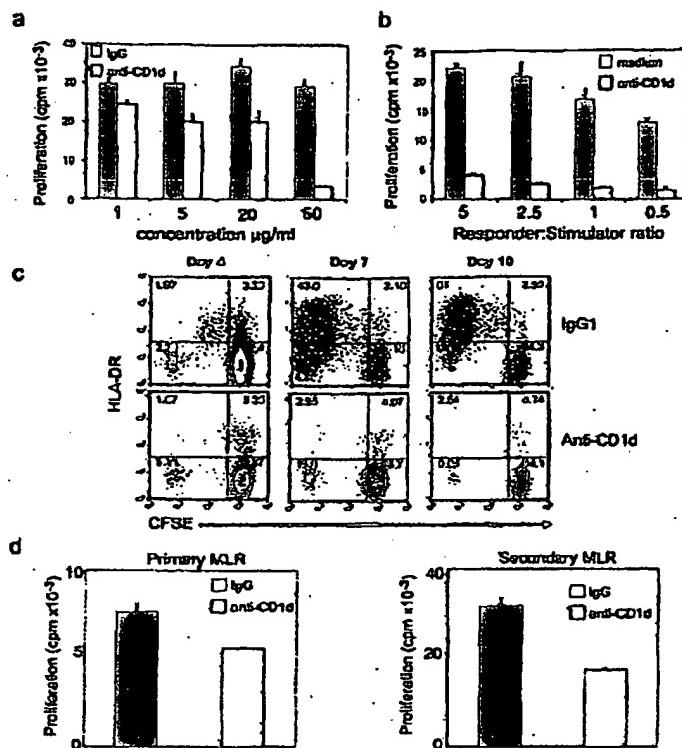
or enhancement of an inhibitory effect. To discriminate between these two effects, MLR were performed using iNKT cell-replete or cell-depleted responders in the presence of anti-CD1d, anti-HLA class II, or Ig isotype (Fig. 6). As expected, the iNKT cell-replete MLR was inhibited by anti-CD1d and by anti-HLA class II mAbs either alone or in combination. iNKT cell depletion resulted in reduction of proliferation but the presence of anti-CD1d in the iNKT cell-depleted MLR did not result in any further significant reduction of proliferation. These results indicate that CD1d is required for the iNKT cell-mediated effect on MLR.

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FIGURE 5. Role of CD1d in the MLR. *a*, MLR were set up at a R:S ratio of 1:2 in the presence of varying concentrations of anti-CD1d or IgG. At an anti-CD1d concentration of as low as 5 $\mu\text{g}/\text{ml}$, MLR reactivity was significantly reduced. *b*, MLR performed in the presence of anti-CD1d 40 $\mu\text{g}/\text{ml}$ was significantly inhibited at varying R:S ratios as compared with MLR treated with medium only. *c*, MLR were performed in the presence of anti-CD1d or IgG (40 $\mu\text{g}/\text{ml}$ each). Proliferation and activation as assessed by CFSE and HLA-DR staining, respectively, were monitored at different time points by flow cytometry. Significant reductions in both proliferation and activation of T cells were observed over time in the presence of anti-CD1d as compared with anti-IgG. *d*, Proliferation was measured in primary 4-day MLR set up at a R:S ratio of 1:2 and treated with either anti-CD1d or IgG isotype (20 $\mu\text{g}/\text{ml}$ each). After 2 days of rest, equal numbers of T cells were placed in a secondary MLR against the same stimulators at a R:S ratio of 1:2 but without mAb. Comparison of the primary with the secondary MLR revealed that CD1d treatment reduces T cell proliferation in the secondary as well as the primary MLR. Each result is representative of three independent MLR.



Discussion

Invariant NKT cells are emerging as an important subset of immunoregulatory cells. Unlike the exclusively suppressive immunomodulation mediated by the CD4 $^{+}$ CD25 $^{+}$ subset of T cells (23), they either enhance or inhibit an Ag-specific immune response through direct interaction with APC (1). This is the first report dissecting the role of human iNKT cells (as defined by the coexpression of the TCR V α 24 and V β 11 chains), CD1d (the restricting element of iNKT cells), and α GC (the iNKT cell ligand) in the alloresponse. Our results suggest that the iNKT cell/CD1d axis positively regulates the alloresponse in humans. This idea is consistent with an earlier report documenting the ability of human iNKT cell clones to drive maturation of DC that can subsequently support efficient alloreactive responses (24). It is also consistent with studies showing that like the alloresponse, other T δ responses against a variety of pathogens (for example, CTL responses against viruses) can diminish in the absence of iNKT cells or CD1d (25, 26). Conversely, in the presence of α GC, T δ innate and adaptive immune responses are considerably enhanced in an iNKT cell- and CD1d-dependent manner (6, 7).

Our data contrast with a number of *in vivo* studies in which iNKT cells appear to inhibit rather than enhance the alloresponse. In murine models of acute GVHD involving myeloablative irradiation of the host, animals that receive α GC injections at the time of irradiation are protected from acute GVHD (10–12). This protective effect of α GC requires the presence of host iNKT cells and CD1d and is mediated by IL-4 secreted by host cells (10, 11). In our studies, in an attempt to mimic this *in vivo* set up, the stimulators (PBMC) of the MLR were irradiated and pulsed with α GC.

However, we consistently observed increased reactivity of the MLR both in terms of proliferation and IFN- γ secretion, an effect that was iNKT cell-dependent. Crucially, no IL-4 was detected at any time point tested during a 4-day MLR. This is in line with the observation that administration of α GC-loaded DC to normal human subjects is associated with increased IFN- γ and IL-12 but decreased IL-4 levels in the serum (27).

A second group of studies have reported that protection from lethal acute GVHD in the context of a nonmyeloablative, tolerance-inducing regimen is associated with persistence of CD1d/ α GC tetramer-positive host iNKT cells (13, 14). Similarly, donor-derived NKT cells (identified as NK1.1 $^{+}$ T cells in C57BL/6 and DX5 $^{+}$ cells in BALB/c mice) protect from lethal acute GVHD after their adoptive transfer to a lethally irradiated host (16, 28). IL-4 and IL-10 seem to be the cytokine mediators of these effects (16, 28). However, NK1.1 $^{+}$ cells were not implicated as being the regulatory T cells that protect from acute GVHD following donor lymphocyte infusion (29, 30).

By performing MLR in the presence and absence of human peripheral blood iNKT cells, we established the importance of responder iNKT cells in enhancing the *in vitro* alloresponse in steady state and without exogenous pharmacological activation. This effect was documented by the specific decrease in proliferation and IFN- γ production in iNKT cell-depleted MLR and by restoration of these parameters upon adding back highly purified fresh iNKT cells. Furthermore, the effect of iNKT cells on the alloresponse was clearly CD1d-dependent. Interestingly, the degree of inhibition of the MLR upon iNKT cell depletion was remarkably uniform for a given responder tested against a panel of stimulators but

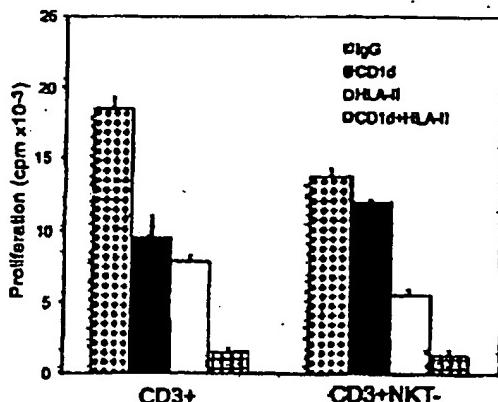


FIGURE 6. CD1d is required for the iNKT cell-mediated effect on MLR. iNKT cell-deplete and cell-depleted MLR were set up in the presence of IgG, anti-CD1d, anti-HLA class II (at 20 μ g/ml each), or a combination of anti-CD1d and anti-HLA. Reactivity, as expected, was reduced in the absence of iNKT cells. Anti-CD1d and anti-HLA class II treatment of iNKT cell-deplete MLR inhibited reactivity by 48 and 57%, respectively. Treatment of the iNKT cell-deplete MLR with anti-CD1d did not have any significant effect compared with IgG treatment (12% inhibition), whereas anti-HLA class II blocking, as in the iNKT cell-deplete MLR, resulted in a significant (60%) reduction of proliferation. Data are representative of three independent MJR.

it varied between responders (Fig. 3*a*, right). This could reflect the variable numbers of TCR Va24 iNKT cells that were not removed by our iNKT cell depletion strategy (see Materials and Methods).

It is not clear why iNKT cells have an enhancing effect in the human *in vitro* alloresponse and the opposite effect in the murine *in vivo* alloresponse. It is possible that because only 50–70% of murine bone marrow and spleen NK1.1⁺ T cells are iNKT cells, as defined by CD1d/oGC tetramer staining (4), NK1.1 marks two groups of CD1d-restricted subsets of regulatory T cells. A subset of cells that may or may not express NK1.1 but are stained with the CD1d/oGC tetramer (iNKT cells) and a subset of cells that always express NK1.1 but are not stained with the CD1d/oGC tetramer (non-iNKT cells). In support of this, Exley et al. (17) have reported that human CD161⁺ T cells, like their murine NK1.1⁺ counterparts, are enriched in bone marrow, secrete IL-4, and suppress the MLR (17). However, these CD161⁺ T cell lines were devoid of iNKT cells. Indeed, only 2 of 12 lines contained any measurable numbers of TCR Va24⁺Vβ11⁺ NKT cells (17).

The cell and cytokine networks responsible for the enhancing effect of iNKT cells in the course of an Ag-specific immune response have been studied in *in vivo* models of microbial infections (31–33). Initial activation of iNKT cells requires a CD1d-dependent cell contact with the APC (1). This leads to increased IFN- γ production by iNKT cells, which in turn enhances the Ag-presenting capability of APC. This positive feedback loop of iNKT cell and DC activation is dependent on DC- and pathogen-derived ligands structurally resembling oGC (31–33). Our data support the existence of a similar network steering the enhancing effect of iNKT cells in the context of the human alloresponse in which iNKT cells can be activated by both exogenous and natural ligands. The structure of the natural ligands driving activation of iNKT cells in the context of the alloresponse remains to be determined.

Because of their flexible immunomodulatory role, iNKT cells hold promise for manipulation and therapeutic use in clinic including modulation of acute GVHD. It is not clear whether addition or

depletion or activation or inactivation of iNKT cells would be beneficial; *in vivo* murine studies would favor adoptive transfer of iNKT cells (13, 14) or treatment of the host with oGC (11), whereas human *in vitro* studies (24 and this work) would favor iNKT cell depletion of donor T cells. Studies of the dynamics of iNKT cell activation in the setting of human acute GVHD will be beneficial in understanding more on the role of iNKT cells *in vivo* and how to exploit them for therapy.

In conclusion, we have shown that iNKT cells are naturally activated during the course of *in vitro* human alloreactivity in a CD1d-dependent manner and although not essential they contribute to the mounting of an efficient alloresponse.

Disclosures

The authors have no financial conflict of interest.

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